



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

JACOTOT et al Atty. Ref.: 1721-90; Confirmation No. 6314
Appl. No. 10/533,642 TC/A.U. To be assigned
Filed: May 3, 2005 Examiner: To be assigned

For: METHOD FOR SCREENING MODULATORS OF MITOCHONDRIAL
FUNCTIONING AND NEW MODULATORS OBTAINED

* * * * *

January 8, 2008

Mail Stop PCT
Commissioner for Patents
Office of PCT Legal Administration
P.O. Box 1450
Alexandria, VA 22313-1450

Attention: PCT Legal Office

Sir:

RENEWED PETITION UNDER 37 CFR 1.47(a)

The present Renewed Petition and attached further evidence is being filed in response to the Decision mailed November 8, 2007.

Grant of the present Petition under Rule 47(a) to accept the previously submitted Declaration signed by inventor Etienne Jacotot and inventor Herve Lecoeur, but not signed by inventor Dominique Rebouillat, who refuses to sign the Declaration.

The last known address of the non-signing inventor, Dominique Rebouillat, is 5, Villa de l'Astrolabe, 75015 Paris, FRANCE.

The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed

JACOTOT et al
Appl. No. 10/533,642
January 8, 2008
RENEWED PETITION UNDER 37 CFR 1.47(a)

herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14 1140.

The attached Second Declaration of Chantal PEAUCHELLE (and attached exhibits) is submitted as factual proof that the missing inventor received a request to execute a new Declaration, with a copy of the originally-filed specification and Preliminary Amendment, and has to date failed to return an executed copy of the new Declaration.

Grant of the present Petition and passage of the present application to OIPE, or other appropriate Office, for further processing for examination are requested.

The Office is requested to contact the undersigned, preferably by telephone, in the event anything further is required for grant of the present Renewed Petition.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: /B. J. Sadoff/
B. J. Sadoff
Reg. No. 36,663

BJS:
901 North Glebe Road, 11th Floor
Arlington, VA 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

En provenance de :

Monsieur Dominique Rebouillat
5 Villa de l'Assistance
75015 PARIS

Présentation le :

Distribution le : 16/12/07

Signature du destinataire ou du mandataire
(Précisez nom et prénom)



RCS PARIS 356 000 000

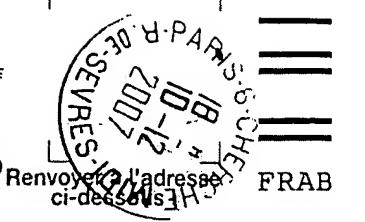
RECOMMANDÉ :
AVIS DE RÉCEPTION

Numéro de l'envoi : 1A 008 461 4997 0

CP/BB 60815-2230



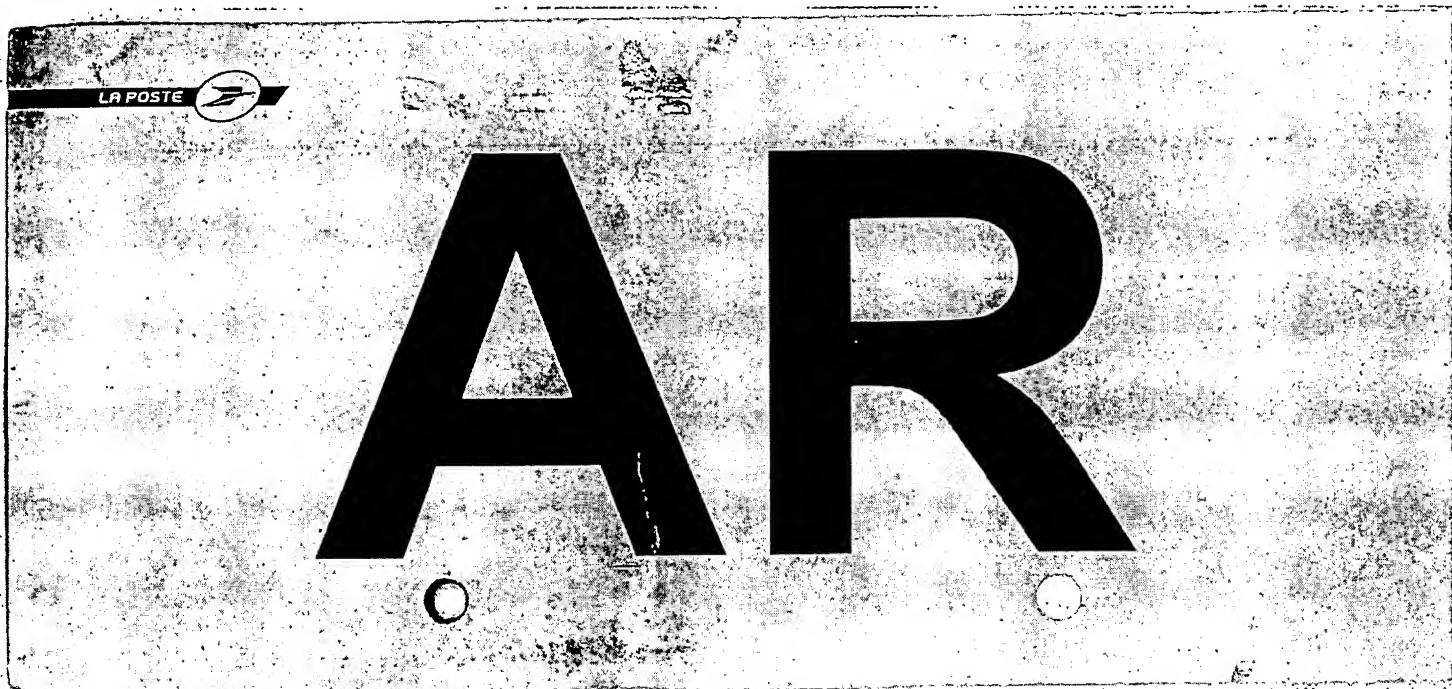
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Colonel Anoukane Aïto
3 Avenue Brégançon
75116 Paris

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



In re Patent Application of

WACOTOT et al

Serial No. **10/533,642**

Filed: May 3, 2005

Atty. Ref.: **1721-90**

Group: **To be assigned**

Examiner: **To be assigned**

For: **METHOD FOR SCREENING MODULATORS OF
MITOCHONDRIAL FUNCTIONING AND NEW
MODULATORS OBTAINED**

* * * * *

Mail Stop PCT
Commissioner for Patents
Office of PCT Legal Administration
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

SECOND DECLARATION OF CHANTAL PEAUCHELLE

I, Chantal PEAUCHELLE, do hereby declare and say as follows:

1. I am a partner in the patent firm of Cabinet ARMENGAUD AINE, 3 Avenue BUGEAUD, 75116 Paris, FRANCE. I am a European and French patent attorney.
2. I am conversant with the French and English languages.
3. I represent the applicant Theraptosis S.A., Biocitech Bâtiment Lavoisier, 102 route de Noisy, 93230 Romainville, France of the International Application No. PCT/EP03/12056.
3. The last known address of Dominique Rebouillat is 5 villa de l'astrolabe, 75015 Paris.
4. I have reviewed the Decision On Petition mailed November 8, 2007 in the above.
5. As suggested in the Decision, the following is a complete translation of the previously-submitted Exhibit 1 of Declaration of CHANTAL PEAUCHELLE, Exhibit 2 of

Declaration of CHANTAL PEAUCELLE and Exhibit 3 of Declaration of CHANTAL

PEAUCELLE and Exhibit 4 of Declaration of CHANTAL PEAUCELLE:



Exhibit 1

Alain ARMENGAUD AINÉ
Licencié en Sciences

Chantal PEAUCELLE
Docteur de spécialité Chimie

Bernard MICHAUDIERE
Ingénieur ECP - Docteur Ingénieur
Conseil en Propriété Industrielle (BMI)
Mandataire agréé auprès de l'Office
Européen des Brevets et de l'OHIM
(Marques communautaires)

Anne GAILLARDIN
Maîtrise de Droit des Affaires et Fiscale
CESS de Propriété Industrielle
Conseil en Propriété Industrielle (BMI)
Mandataire agréé auprès de l'OHIM

Eric DONNEGER
Maîtrise en Chimie Physique
D.E.A. Génie des Procédés Industriels
Diplôme C.E.I.P.I. - (BMI)

Blandine TARRERE
Ingénieur C.P.E.
Conseil en Propriété Industrielle (BMI)
Mandataire agréé auprès de
l'Office Européen des Brevets

Patrick MONLOUIS
Ingénieur ENSAM
(Option Matériaux)
Diplôme C.E.I.P.I. - (BMI)

Services Administratifs
Annalistes : Colombe ANGER
Joëlle PIERRE-DOUBLET
Comptable : Catherine FOIN
Marques, Dessins et Modèles :
Caroline de BONTIN

CABINET ARMENGAUD AINÉ FONDÉ EN 1836

Membre d'une association agréée - Le règlement par chèque est accepté.
S.C.P. au capital de 20 123,27 € - R.C.S. Paris D 392 004 404 - Siret N° 392 004 404 00019 - APE N° 741 A
Brevets d'invention - Marques de Fabrique - Dessins et Modèles
Procès en contrefaçon - Recherches d'antériorités - Consultations

3, AVENUE BUGEAUD, 75116 PARIS - ☎ 01 45 53 05 50
Téléfax : 01 45 53 80 21 / 01 47 55 12 96 - E-mail : info@armengaud.fr

Monsieur Dominique REBOUILLAT
5, Villa de l'Astrolabe
75015 Paris

Paris, March 26, 2007

Y/ref. : B3

O/ref. : CP/BB 60815-2230

US patent application 10/533 642 - National phase of
PCT/EP03/12056 filed on October 2, 2003- In the name of
THERAPTOsis SA - "Method for screening modulators of
mitochondrial functioning and new modulators obtained" -
Inv. Jacotot et al - US Priorities 60/415,092 of October 2, 2002
and US 60/472,725 of May 23, 2003

By registered mail - Declaration reminder

Dear Sirs,

Please find enclosed a Declaration for execution by yourself regarding the
application. We remind you that the deadline for filing the document expires on
May 6, 2007.

Would you please send us back this document after execution as soon as
possible?

Yours sincerely,

Chantal PEAUCELLE

Encl: Declaration

SECOND DECLARATION OF CHANTAL PEAUCELLE

Exhibit 2 of Declaration of CHANTAL PEAUCELLE

Addressee

Monsieur Dominique Rebouillat
5 Villa de l'Astrolobe
75015 Paris

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CERTIFIED MAIL RECEIPT **TO BE RETAINED BY THE SENDER**

Exhibit 3 of Declaration of CHANTAL PEAUCELLE

Sender's address:

~~Monsieur Dominique Rebouillat
5 Villa de l'Astrolobe
75015 Paris~~

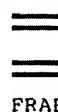
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3, Avenue Bugeaud
75116 PARIS

JACOTOT et al
10/533,642
SECOND DECLARATION OF CHANTAL PEAUCELLE

Alain ARMENGAUD AINÉ
Licencié en Sciences

Chantal PEAUCELLE
Docteur de spécialité CNRS

Bernard MICHARDIÈRE
Ingénieur ECP - Docteur Ingénieur

Conseils en Propriétés Industrielles (BPI)

Mandataire agréé auprès de l'Ofice

Européen des Brevets et de l'OMI

Marques communautaires

Anne GAILLARDIN
Maîtresse de Droit des Affaires et Faculté
DESS de Propriété Industrielle
Conseil en Propriétés Industrielles (BPI)

Mandataire agréée auprès de l'Ofite

Eric DONNEGER
Maîtresse de Droit Physique
D.E.A. Génie des Procédés Industriels
Diplôme C.E.I.P. - (BPI)

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Ingénierie C.P.E.
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Ingénieur ENSAM
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Docteur C.E.I.P. - (BPI)

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Comptable : Catherine FOIN
Marques, Dessins et Modèles :
Caroline de BONTIN

REMINDER

URGENT

Y/ref. : B3

O/ref. : CP/BB 60815-2230

US patent application 10/533 642 - National phase of
PCT/EP03/12056 filed on October 2, 2003- In the name of
THERAPTOSIS SA - "Method for screening modulators of
mitochondrial functioning and new modulators obtained" -
Inv. Jacotot et al - US Priorities 60/415,092 of October 2, 2002
and US 60/472,725 of May 23, 2003

By registered mail - Declaration reminder

Dear Sirs,

Please find enclosed a Declaration for execution by yourself regarding the application. We remind you that the deadline for filing the document expires on May 6, 2007.

Would you please send us back this document after execution as soon as possible?

Yours sincerely,

Chantal PEAUCELLE

Encl: Declaration

6. Attached hereto is a copy of a letter mailed to Dominique Rebouillat on December 6, 2007, by registered mail with acknowledgement of receipt transmitting a copy of the inventor's Declaration, the specification as filed, the Preliminary Amendment filed May 3, 2005 and the Sequence Listing (also attached). Also attached is an English translation of the attached letter of December 6, 2007 to Dominique Rebouillat and an English translation of the acknowledgement receipt form executed by Dominique Rebouillat on December 10, 2007.

7. To date, a copy of the Declaration executed by Dominique Rebouillat has not been received.

I hereby declare that all statements made herein of my/our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this 8 day of January, 2008.

(Signature) Chantal

JACOTOT et al
10/533,642
SECOND DECLARATION OF CHANTAL PEAUCELLE

(Print) Chantal PEAUCELLE

(Title) French and European Patent Attorney

Chantal PEAUCELLE
Docteur de spécialité Chimie

Bernard MICHARDIÈRE
Ingénieur ECP - Docteur Ingénieur

Conseils en Propriété Industrielle (BM)
Mandataires agréés auprès de l'Office
Européen des Brevets et de l'OHMI
(Marques communautaires)

Anne GAILLARDIN
Maîtrise de Droit des Affaires et Fiscalité
DESS de Propriété Industrielle
Conseil en Propriété Industrielle (M)
Mandataire agréée auprès de l'OHMI

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D.E.A. Génie des Procédés Industriels
Diplômé C.E.I.P.I. - (BM)

Blandine TARRERE
Ingénieur C.P.E.
Conseil en Propriété Industrielle (B)
Mandataire agréée auprès de
l'Office Européen des Brevets

Patrick MONLOUIS
Ingénieur ENSAM
(Option Matériaux)
Diplômé C.E.I.P.I. - (B)

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Caroline de BONTIN

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Membre d'une association agréée - Le règlement par chèque est accepté.
S.C.P. au capital de 20 123,27 € - R.C.S. Paris D 392 004 404 - Siret N° 392 004 404 00019 - APE N° 741 A
Brevets d'invention - Marques de Fabrique - Dessins et Modèles
Procès en contrefaçon - Recherches d'antériorités - Consultations

3, AVENUE BUGEAUD, 75116 PARIS - ☎ 01 45 53 05 50

Téléfax : 01 45 53 80 21 / 01 47 55 12 96 - E-mail : info@armengaud.fr

Monsieur Dominique REBOUILLET
5, Villa de l'Astrolabe
75015 Paris

Paris, le 6 décembre 2007

V/réf. : B3

N/réf. : CP/BB 60815-2230

Demande de brevet US 10/533,642 correspondant au
PCT/EP03/12056 du 2 octobre 2003 - Au nom de Theraptosis SA -
"Method for screening modulators of mitochondrial functioning and
new modulators obtained" - Inv. Jacotot et al - Priorités US
60/415,092 du 2 octobre 2, 2002 et US 60/472,725 du 23 Mai 2003

Recommandé AR

Cher Monsieur,

Nous vous avons adressé le 26 mars 2007 un courrier avec une Déclaration aux
fins de signature en vue de son dépôt auprès de l'Office des Brevets américain.

Pour satisfaire à la demande de l'Office des brevets américain, nous vous
adressons à nouveau un exemplaire de ce document auquel est attaché copie du
texte de la demande US en référence tel que déposé, de l'amendement
préliminaire du 3 mai 2005 et du listage de séquences.

Nous vous remercions par avance de nous le retourner après signature pour le
20 décembre 2007 au plus tard.

Nous sommes à votre disposition pour vous fournir des compléments
d'informations si souhaité.

Veuillez agréer, Cher Monsieur, nos salutations distinguées.


Chantal PEAUCELLE

PJ. : Déclaration + texte US avec amendement et listage de séquences

Monsieur Dominique REBOUILLAT
5, Villa de l'Astrolabe
75015 Paris

Paris, December 6, 2007

Y/ref. : B3
O/ref. : CP/BB 60815-2230

US patent application 10/533 642 - National phase of PCT/EP03/12056
filed on October 2, 2003- In the name of THERAPTOSIS SA - "Method
for screening modulators of mitochondrial functioning and new modulators
obtained" - Inv. Jacotot et al - US Priorities 60/415,092 of October 2,
2002 and US 60/472,725 of May 23, 2003

By registered mail with acknowledgement receipt form

Dear Sirs,

We sent you on March 26, 2007 a mail with a Declaration for execution for filing at the USPTO.

To satisfy the USPTO's requirement, we send you again a new exemplar of this document to which is appended a copy of the specification of the above mentioned US application such as filed, the preliminary amendment of May 3, 2005 and the sequence listing.

We thank you in advance to send it us back after execution for December 20, 2007 at latest.

We remain at your disposal to give you additional information, if desired.

Very truly yours,

Chantal PEAUCELLE

Encl.: Declaration + US specification with amendment and sequence listing

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named Inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the Invention entitled:

METHOD FOR SCREENING MODULATORS OF MITOCHONDRIAL FUNCTIONING AND NEW MODULATORS OBTAINED

the specification of which (check applicable box(es)):

is attached hereto

was filed on May 3, 2005 as U.S. Application Serial No. 10/533,642 (Atty. Dkt. No. 1721-90)

was filed as PCT International application No. PCT/EP2003/012056 on 2 October 2003

and (if applicable to U.S. or PCT application) was amended on 3 May 2005

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.		
Application Number 60/415,092 60/472,725	Date/Month/Year Filed 2 October 2002 23 May 2003	

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below:

Prior U.S./PCT Application(s): Application Serial No. PCT/EP2003/012056	Day/Month/Year Filed 2 October 2003	Status: patented pending, abandoned
---	--	--

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint Nixon & Vanderhye P.C., telephone number 703-816-4000 (to whom all communications are to be directed) and the attorneys of: **Customer Number 23117**, individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent. I also authorize Nixon & Vanderhye to add or delete attorneys from that Customer Number, and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

1.	Inventor's Signature:				Date:
	Inventor:	Etienne (first)	MI	Jacotot (last)	France (citizenship)
	Residence: (city)	Paris		(state/country)	France
	Mailing Address:	171, Rue Lecourbe, Paris, France			
	(Zip Code)	F-75015			
2.	Inventor's Signature:				Date:
	Inventor:	Herve (first)	MI	Lecoeur (last)	France (citizenship)
	Residence: (city)	Paris		(state/country)	France
	Mailing Address:	113, Rue Didot, Paris, France			
	(Zip Code)	F-75014			

See attached sheet(s) for additional inventor(s) information!!

3.	Inventor's Signature:				Date:	
	Inventor:	Dominique (first)	MI	Rebouillat (last)	France	
	Residence: (city)	Paris		(state/country)	(citizenship)	
	Mailing Address:	5, Villa de l'Astrelabe, Paris, France				
	(Zip Code)	F-75015				
4.	Inventor's Signature:				Date:	
	Inventor:	(first)	MI	(last)	(citizenship)	
	Residence: (city)			(state/country)		
	Mailing Address:					
	(Zip Code)					
5.	Inventor's Signature:				Date:	
	Inventor:	(first)	MI	(last)	(citizenship)	
	Residence: (city)			(state/country)		
	Mailing Address:					
	(Zip Code)					
6.	Inventor's Signature:				Date:	
	Inventor:	(first)	MI	(last)	(citizenship)	
	Residence: (city)			(state/country)		
	Mailing Address:					
	(Zip Code)					

FOR ADDITIONAL INVENTORS, check box [] and attach sheet with same information and signature and date for each.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

JACOTOT ET AL.

Atty. Ref.: 1721-90

Serial No. **To be assigned**

Group: **To be assigned**

National Phase of : PCT/EP2003/012056

International Filing Date: 2 October 2003

Filed: **May 3, 2005**

Examiner: **To be assigned**

For: **METHOD FOR SCREENING MODULATORS OF
MITOCHONDRIAL FUNCTIONING AND NEW
MODULATORS OBTAINED**

*) **May 3, 2005**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend as follows:

AMENDMENTS TO THE ABSTRACT

Please insert the Abstract of the Disclosure which is on the attached sheet.

ABSTRACT

The invention relates to a method for screening modulators of mitochondrial function comprising adding a compound to be tested in a purified, isolated mitochondria preparation and simultaneously using fluorimetric analysis of mitochondrial morphology, and especially real-time fluorimetric analysis, combining analysis of morphometric parameters (SSC/FSC parameters) with analysis of membrane integrity by dye fluorescence. Application to the obtention of peptides which induce mitochondrial membrane permeabilization.

AMENDMENTS TO THE SPECIFICATION:

Page 1, after the title insert the following:

This application is the US national phase of international application
PCT/EP2003/012056 filed **2 October 2003** which designated the U.S. and claims
benefit of **U.S. Provisional Applications 60/415,092**, filed **2 October 2002**, and
60/4742,725, filed **23 May 2003**, the entire contents of each of which are hereby
incorporated by reference.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. (Original) A method for screening modulators of mitochondrial function comprising adding a compound to be tested in a purified, isolated mitochondria preparation and simultaneously using fluorimetric analysis of mitochondrial morphology, and especially real-time fluorimetric analysis, combining analysis of morphometric parameters (SSC/FSC parameters) with analysis of membrane integrity by dye fluorescence.
2. (Original) The method of claim 1, wherein the analysis of the membrane integrity is performed using the JC-1 dye to characterize mitochondrial transmembrane potential Aym and study any change of the mitochondrial membrane permeability.
3. (Currently Amended) The method of claim 1-~~or 2~~, wherein the mechanism of mitochondrial membrane permeabilization is further characterized using pharmacological inducers or modulators of mitochondrial permeability such as Calcium, CCCP, Alamethicin, Bongrekic acid, ruthenium red, cyclosporin A, DIDS.
4. (Currently Amended) The method of claim 1~~anyone of claims 1 to 3~~, further comprising studying endogenous or xenogenic mitochondrial regulators such as Bcl-2 family members and respiratory inhibitors on PTP-related MMP.
5. (Currently Amended) The method of claim 1~~anyone of claims 1 to 3~~, further comprising identifying compounds inducing MMP or preventing MMP induced by other agents, alone or in combination.

6. (Currently Amended) The method of claim 1~~anyone of claims 1 to 5~~, further comprising designing and defining new agents aiming at modulating MMP.

7. (Currently Amended) The method of claim 1~~anyone of claims 1 to 6~~, further comprising defining and manufacturing reagents or kits of reagent to put the method in use.

8. (Currently Amended) The method according to claim 1~~anyone of claims 1 to 4~~, further comprising characterizing mitochondrial activity in specific mitochondria, to be used to ascertain mitochondrial function in mitochondria from various tissues of physiological or pathological status, or pathology sources such as tumors.

9. (Currently Amended) The method according to claim 1~~anyone of claims 1 to 4~~, further comprising the diagnosis or characterization of mitochondrial function in patients with diseases, and especially genetic diseases or metabolic diseases.

10. (Currently Amended) Agents identified by using method according to claim 1~~claims 1 to 4~~, and shown to be able to modulate MMP on isolated mitochondria.

11. (Currently Amended) Peptides according to claim 10, having MMP modulating potency, selected in the group comprising : ATLSALLAALRRIQRA (SEQ ID NO:1¹) RKKRRQRRRGATLSALLAALRRIQRA (SEQ ID NO:2²) RKKRRQRRRCGGLETRTETWMSSEGAWKQIQKVETWALRH (SEQ ID NO:3³) RKKRRQRRRCGGLANKKGAWLDSTKATRYLVKTESWILRN (SEQ ID NO:4⁴) GG*CRGDMFG*CGGLLFIHFRIGSRHSRIG (SEQ ID NO:5⁵) RIEIWILRH (SEQ ID NO:6⁶) RIAIWILRH (SEQ ID NO:7⁷) RKKRRQRRRGRIEIWILRH (SEQ ID NO:8⁸) RKKRRQRRRGRIAIWILRH (SEQ ID NO:9⁹) EHWSYWLRPGGGLLFIHFRIGCRHSRIG (SEQ ID NO:10¹⁰)

EHWSYWL RP GGGGGSLL FIHFRIGCRHSRIG (SEQ ID NO:11¹¹) and their derivatives.

Said said peptides advantageously optionally further comprise in the C-terminal and N terminal position a stabilizing group such as an amide alkyl or acyl group and a marker or linking group, such as the biotinyl group. They can, and also include optionally L- or D-aminoacids retro-inverso, reduced peptidic backbone, or being translated into pseudo peptides.

The invention also relates to said peptides bound to a peptidic delivery system, and optionally comprising a linker between said peptides and delivery system. This peptidic delivery system could advantageously be an extra cellular or intracellular targeting sequence, an antibody or a fragment thereof (ScFv). The linker can be a sequence allowing the peptide to adopt an helical structure independently of the peptidic delivery system, and can be formed by 2 to 6 aminoacids such as alanine or glycine, or a disulfide bridge, or any such means as known by a man skilled in the art considering the state of the art knowledge. Said peptide could also be modified by insertions, deletions or mutations which conserve mitochondrial modulation potency.

12. (Currently Amended) Peptides obtained by analogy to peptides described in claim 11, presenting features characteristics of a MMP modulating function, defined as:
- containing at least 8 amino acids, and up to 50 amino acids - at least a part of the peptide structure is an amphipathic alpha helix, - 2, 3 or 4 amino acids are positively charged (lysine [K] or arginine [R]) and are located on the same side of the helix, - when the helix is projected using helical wheel representation, the R and/or K amino

acids form a cluster (see figure 15), when added to purified, isolated, mitochondria they induce changes (ultrastructural or membrane permeability).

Example of such peptides are selected below:

~~RKKRRQRRRGGGAWKHAQRIEIWLRH (SEQ ID N°12)~~

~~RKKRRQRRRGGGAWKHAQRIETWLRH (SEQ ID N°13)~~

~~RKKRRQRRRGGGAWKHAQRVESWLRN (SEQ ID N°14)~~

~~RKKRRQRRRGGGAWKRACRMETWLRH (SEQ ID N°15)~~

~~RKKRRQRRRGGGAWKQIQKVETWALRH (SEQ ID N°16)~~

~~RKKRRQRRRGGGAWRQVEKVETWALRH (SEQ ID N°17)~~

~~RKKRRQRRRGGGAWKHAQRIAIWLRH (SEQ ID N°18)~~ AWKHAQRIAIWLRH (SEQ

~~ID N°19)~~ GG*CRGDMFG*CGGRIEIWLRH (SEQ ID N°20)

~~GG*CRGDMFG*CGGRIAIWLRH (SEQ ID N°21)~~ GG*CGRGDSPG*CGGRIEIWLRH

~~(SEQ ID N°22)~~ GG*CGRGDSPG*CGGRIAIWLRH (SEQ ID N°23) Where *C = cysteine

~~engaged in cycling disulfide bridge EHWSYWLRPGGGRIEIWLRH (SEQ ID N°24)~~

~~EHWSYWLRPGGGGRIAIWLRH (SEQ ID N°25)~~ EHWSYWLRPGGGGGGSRIEIWLRH

~~(SEQ ID N°26)~~ EHWSYWLRPGGGGGGSRIAIWLRH (SEQ ID N°27)

~~EHWSYWLRPGGGGGSGAWKHAQRIEIWLRH (SEQ ID N°28)~~

~~EHWSYWLRPGGGGGSGAWKHAQRIAIWLRH (SEQ ID N°29)~~

~~EHWSYWLRPGGGGLLFIHFKIGCKHSKIG (SEQ ID N°30)~~

~~EHWSYWLRPGGGGGSLLFIHFKIGCKHSKIG (SEQ ID N°31)~~

~~EHWSYWLRPGGGGLLFIHFRIIGSRHSRIG (SEQ ID N°32)~~

~~EHWSYWLRPGGGGGSLLFIHFRIIGSRHSRIG (SEQ ID N°33)~~

~~EHWSYWLRPGGGGLLFIHFKIGSKHSKIG (SEQ ID N°34)~~

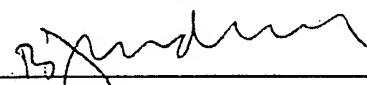
~~EEHWSYWLPRGGGGGSLLFIHKIGSKHSKIG (SEQ ID N°35)~~ in which the W6 residue
can be replaced by the D-aminoacid dW. These peptide are claimed along with their
derivatives as defined in claim 10.

REMARKS

The claims have been amended to eliminate multiple dependent claims, without prejudice. The specification has been amended to include a cross-reference to the parent applications and to add an Abstract of the Invention.

Respectfully submitted,

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"Method for screening modulators of mitochondrial functioning and new modulators obtained"

The invention relates to a method for screening modulators of mitochondrial functioning and to the new modulators obtained.

During the last ten years, an intensive international research revealed the key role of mitochondria in apoptosis and other related forms of programmed cell death. Although mitochondrion is not the only organelle able to regulate apoptotic cellular demise, a large array of experimental evidences suggest that mitochondria is a critical component of the "central executioner" of apoptosis. As a result, the study of the regulation of mitochondrial function *in vitro*, using isolated mitochondria, are of a great interest both to decipher cell death mechanisms and to identify new therapeutic drugs.

Mitochondrial metabolism and permeability state of mitochondrial membranes are essential checkpoints for cell life and death decisions, and indeed, during mammalian programmed cell death, mitochondria are subjected to different structural and functional modifications. Experimentally, agents that block respiratory chain trigger bio-emergent catastrophe and molecules able to modify mitochondrial membrane permeability are efficient regulators of cell death including necrotic, apoptotic and autophagic types of cell death. During programmed cell death, also called apoptosis, mitochondrial modifications usually include: matrix swelling, outer membrane permeabilization (OMP) to intermembrane apoptosis effectors (such as cytochrome c, apoptosis-inducing factor, endoG, and Smac/Diablo), as well as dissipation of the inner transmembrane potential ($\Delta\psi_m$). The exact hierarchy of these events is still a matter of debate and may depend both on cell type and cell death pathways. A well-known phenomenon often responsible for this mitochondrial membrane permeabilization (MMP) is the Mitochondrial Permeability

Transition (MPT). MPT can lead to a rapid mitochondrial swelling (i.e. an increase in mitochondrial size and a decrease in the organelles' granularity), to the rupture of the outer membrane, and can induce a dissipation of the 5 mitochondrial transmembrane potential ($\Delta\psi_m$).

Pro- and anti-apoptotic members of the Bcl-2 family regulate inner and outer mitochondrial membrane permeabilization (MMP) through interactions with the adenine nucleotide translocator (ANT; in the inner membrane, IM), the 10 voltage-dependent anion channel (VDAC; in the outer membrane, OM), and/or through autonomous channel-forming activities. ANT and VDAC are major components of the permeability transition pore (PTP), a polyprotein structure organized at sites at which the two mitochondrial membranes are apposed. The PTP is 15 a non-specific pore, permeant to any molecule of < 1.5 kDa, which opens in the inner mitochondrial membrane under conditions of oxidative stress, high Ca^{2+} or low ATP concentrations. Opening of the PTP causes massive swelling of mitochondria, rupture of the outer membrane and release of 20 intermembrane components that induce apoptosis. In addition mitochondria become depolarized causing inhibition of oxidative phosphorylation and stimulation of ATP hydrolysis. Pore opening is inhibited by cyclosporin A or derivatives of 25 this compound that bind to mitochondrial cyclophilin (peptidyl-prolyl-cis-trans-isomerase).

The inventors have found that morphological and $\Delta\psi_m$ changes of isolated mitochondria can be quantified simultaneously in real-time by FCM (flow cytometry) analysis on a per-mitochondrion basis. Using this approach, they have 30 developed a method for screening modulators of mitochondrial functioning and have obtained a new family of peptidic inducers of MMP.

The progressive mitochondrial swelling is detected by the significant increase in forward scatter (FSC) and the decrease 35 in the side scatter (SSC) of the organelles. Both observations are significantly correlated with spectrophotometric

absorbance data. Variations in $\Delta\psi_m$ are followed after loading with the JC-1 probe. This cationic lipophilic dye forms J-aggregates in highly energized mitochondria (emitting red fluorescence), whereas only present in monomers in the matrix of low $\Delta\psi_m$ organelles (associated with green fluorescence).
5 The drop of the $\Delta\psi_m$ is followed by the progressive decrease in JC-1 red fluorescence and in the JC-1 red / green fluorescence ratio, both resulting from the dissociation of J-aggregates.

10 The invention thus relates to a method for screening modulators of mitochondrial function by real-time flow cytometry analysis, and is characterized in the Examples 1 to 3.

15 Example 1 describes the characterization of mitochondrial preparation by FCM: although purified mouse liver mitochondria are small organelles (1-2 μm), they are detectable by flow cytometry using the $\Delta\psi_m$ -insensitive dye MitoTracker[®] Green, for a purity reaching 95%. Moreover, their functionality can be assessed using the $\Delta\psi_m$ -sensitive dye JC-1: most of these 20 organelles are energetically competent since more than 90% have an elevated $\Delta\psi_m$).

25 Example 2 shows how swelling quantification can be performed on isolated mitochondria, and the advantages of real-time analysis versus fixed time flow cytometry. Indeed, fixed time and real time FCM allow following changes induced by the PTP opener calcium. Real time allows integral calculus to estimate the velocity of these morphological changes.

30 However, for slow changes in mitochondrial morphology such as the protonophore carbonyl cyanide meta-chlorophenylhydrazone (CCCP), real-time is advantageous as it allows to follow a low but significant granularity (SSC) decrease without detectable size (FSC) changes. Indeed, using real-time fluorimetric follow-up of the mean SSC signal and the mean forward scatter (FSC) signal, it appears that CCCP

induces a reduction of SSC that cannot be detected by classical spectrophotometric absorbance analysis.

When PTP-independent mitochondrial swelling is induced by the ion channel forming antibiotic peptide alamethicin, a simultaneous FSC increase and SSC decrease is also observed. Interestingly, with this molecule, SSC variations were less rapid than FSC modifications. Thus, real time analysis allows to better define different PTP modifiers.

Example 3 demonstrates how the area of real-time fluorimetric analysis can be extended to $\Delta\Psi_m$. Real time analysis allows to characterize mitochondria integrity and to differentiate various PTP-dependant and PTP-independent MMP inducers. Indeed, it allows a rapid identification of the mechanistic aspects of Calcium, alamethicin and CCCP, testing these drugs alone or in association with inhibitors such as Bongkretic acid, ruthenium red and Cyclosporin A. As an example of the utility of the method, these analyses indicate that at least in these experimental conditions, $\Delta\Psi_m$ loss does not precede Ca^{2+} induced mitochondrial internal structure remodeling. Thus simultaneous flow cytometry real-time recording of mitochondrial size/structure and $\Delta\Psi_m$ state may be used as a tool to define the hierarchy of MMP events.

Simultaneous detection of SSC and JC1-orange is thus a rapid and acute method to screen and quantify effects of MMP-inhibitors.

As shown in the examples above, this real-time approach is useful for characterization of the mitochondrial membrane permeabilization (MMP) induced by various molecules including peptides and small molecules. Said real-time approach is also useful to study the effect of different inhibitors of the respiratory chain (malonate, antimycin A, KCN, oligomycin, or the protonophore uncoupler CCCP) on MMP.

The invention thus relates to a method for screening modulators of mitochondrial functioning comprising adding a compound to be tested in a purified, isolated mitochondria preparation and simultaneously using a real-time fluorimetric

analysis of mitochondrial morphology combining analysis of morphometric parameters (SSC/FSC parameters) with analysis of membrane integrity by dye fluorescence.

The analysis of the membrane integrity is performed using 5 the JC-1 dye to characterize mitochondrial transmembrane potential $\Delta\psi_m$ and study any change of the mitochondrial membrane permeability.

The mechanism of mitochondrial membrane permeabilization is further characterized using pharmacological inducers or 10 modulators of mitochondrial permeability such as Calcium, CCCp, Alamethicin, Bongkretic acid, ruthenium red, cyclostorin A, DIDS.

Such a method enables to study for example the impact of Bcl-2 family members and respiratory inhibitors on PTP-related 15 MMP, and is also useful for example to identify and design new agents inducing mitochondrial permeability changes, alone or in combination, said compounds being for example chemicals, peptides, biological extracts from human or non human origin (e.g. from mammalian, animal, bacterial, yeast, or plant, 20 either natural or created by genetic engineering).

The invention also relates to the use of said method for designing new agents aiming at modulating MMP.

The invention also concerns a method such as above defined comprising the diagnosis or characterization of 25 mitochondrial function in patients with diseases, and especially genetic diseases or metabolic diseases.

Said above defined methods are also useful to define agents able to cure defects thus revealed, any follow patient evolution.

30 In a specific embodiment, using the above-mentioned real time FCM analysis of mitochondria for screening, the inventors defined mitochondrially active peptides.

The invention thus relates to synthetic peptides which induce mitochondrial membrane permeabilization (MMP) when 35 added to purified, isolated, mitochondria, said peptide being composed of L or D aminoacids, protected at the C terminus by

amide or similar protection, and in N-ter by acyl, alkyl, biotinyl groups, or other modifications.

The invention also relates to said peptides and their derivative as containing simple addition, deletions, or 5 mutations without altering the mitochondrial function.

Peptides according to the invention have the following sequences :

ATLSALLAALRRIQRA (SEQ ID N°1)

RKKRRQRRGGATLSALLAALRRIQRA (SEQ ID N°2)

10 RKKRRQRRCGGLETRTETWMSSEGAWKQIQKVETWALRH (SEQ ID N°3)

RKKRRQRRCGGLANKKGAWLDSKATRYLVKTESWILRN (SEQ ID N°4)

GG*CRGDMFG*CGGLLFIHFRIGSRHSRIG (SEQ ID N°5)

RIEIWILRH (SEQ ID N°6)

RIAIWILRH (SEQ ID N°7)

15 RKKRRQRRGGRIEIWILRH (SEQ ID N°8)

RKKRRQRRGGRIAIWILRH (SEQ ID N°9)

EHWSYWL RP GGGLLFIHFRIGCRHSRIG (SEQ ID N°10)

EHWSYWL RP GGGGSLLFIHFRIGCRHSRIG (SEQ ID N°11)

and their derivatives.

20 In this list, asterisk indicate disulfide bridge between cysteins.

In sequences ID 10 and 11, the W6 residue can advantageously be replaced by the D-aminoacid α W.

Said peptides were shown to induce mitochondrial changes 25 and MMP, and have the capacity to induce apoptosis in cultured cells, and thus have the potential to be used to treat some human diseases.

Said mitochondrial properties of these sequences are shown in Example 4, demonstrating the ability of the technique 30 to detect mitochondrial permeability inducers.

The inventors observed, during the above testing, a set of structural features highly indicative of a potency to act on mitochondrial permeability. This structural particularity can be used for the identification or design of a broad range 35 of peptides modulating MMP.

The invention relates to any peptide containing at least 8 amino acids and until 50 amino acids having the following features:

- at least a part of the peptide structure is an amphipathic alpha-helix,
- 5 - 2, 3 or 4 amino acids are positively charged (lysine [K] or arginine [R]) and are located on the same side of the helix,
- when the helix is projected using helical wheel representation, the R and/or K amino acids form a cluster (see figure 15),
- 10 - when added to purified, isolated, mitochondria they induce changes (ultrastructural or membrane permeability).

15 Examples of peptides presenting these characteristics and having mitochondrial activity are listed below:

RKKRRQRRGGGAWKHAQRIEIWILRH	(SEQ ID N°12)
RKKRRQRRGGGAWKHAQRRIETWILRH	(SEQ ID N°13)
RKKRRQRRGGGAWKHAQRVESWILRN	(SEQ ID N°14)
20 RKKRRQRRGGGAWKRACRMETWILRH	(SEQ ID N°15)
RKKRRQRRGGGAWKQIQKVETWALRH	(SEQ ID N°16)
RKKRRQRRGGGAWRQVEKVETWALRH	(SEQ ID N°17)
RKKRRQRRGGGAWKHAQRIAIWILRH	(SEQ ID N°18)
AWKHAQRIAIWILRH	(SEQ ID N°19)
25 GG*CRGDMFG*CGGRIEIWILRH	(SEQ ID N°20)
GG*CRGDMFG*CGGRIAIWILRH	(SEQ ID N°21)
GG*CGRGDSPG*CGGRIEIWILRH	(SEQ ID N°22)
GG*CGRGDSPG*CGGRIAIWILRH	(SEQ ID N°23)
Where *C = cysteine engaged in cycling disulfide bridge	
30 EHWSYWLPGGGGRIEIWILRH	(SEQ ID N°24)
EHWSYWLPGGGGRIAIWILRH	(SEQ ID N°25)
EHWSYWLPGGGGGSRIEIWILRH	(SEQ ID N°26)
EHWSYWLPGGGGGSRIAIWILRH	(SEQ ID N°27)
EHWSYWLPGGGGGSGAWKHAQRIEIWILRH	(SEQ ID N°28)
35 EHWSYWLPGGGGGSGAWKHAQRIAIWILRH	(SEQ ID N°29)
EHWSYWLPGGGGLFIHFKIGCKHSKIG	(SEQ ID N°30)

EHWSYWL RP GGGGG SLL FI HF KIG CKH SKIG	(SEQ ID N°31)
EHWSYWL RP GGGGLL FI HF RIG SRH SRIG	(SEQ ID N°32)
EHWSYWL RP GGGGG SLL FI HF RIG SRH SRIG	(SEQ ID N°33)
EHWSYWL RP GGGGLL FI HF KIG SKH SKIG	(SEQ ID N°34)
5 EHWSYWL RP GGGGG SLL FI HF KIG SKH SKIG	(SEQ ID N°35)

In which the W6 residue can advantageously be replaced by the D-aminoacid α W.

10 Said peptides advantageously comprise in the C-terminal position a stabilizing group such as an amide group and a marker or linking group, such as the biotinyl group. They can also include L- or D- aminoacids retro-inverso, reduced peptidic backbone, or being translated into pseudo peptides.

15 The invention also relates to said peptides bound to a peptidic delivery system, and optionally comprising a linker between said peptides and delivery system. This peptidic delivery system could advantageously be an extra cellular or intracellular targeting sequence, an antibody or a fragment thereof (ScFv). The linker can be a sequence allowing the peptide to adopt an helical structure independently of the 20 peptidic delivery system, and can be formed by 2 to 6 aminoacids such as alanine or glycine, or a disulfide bridge, or any such means as known by a man skilled in the art considering the state of the art knowledge. Said peptide could also be modified by insertions, deletions or mutations which 25 conserve mitochondrial modulation potency.

Said peptide can be divided in families depending of the target cells for a possible therapeutic use:

30 Group 1: synthetic peptides that induce cell death of various cell types. Potency of these peptides to induce cell apoptosis is shown in Example 5.

RKKRRQRRRGGRIEIWILRH	(SEQ ID N°8)
RKKRRQRRRGGRIAIWILRH	(SEQ ID N°9)
RKKRRQRRRGGAWKHAQRIEIWILRH	(SEQ ID N°36)
RKKRRQRRRGGAWKHAQRRIETWILRH	(SEQ ID N°37)
35 RKKRRQRRRGGAWKHAQRVESWILRN	(SEQ ID N°38)
RKKRRQRRRGGAWKRA CRM ETWILRH	(SEQ ID N°39)

RKKRRQRRRGGGAWKQIQQVETWALRH (SEQ ID N°40)
 RKKRRQRRRGGGAWRQVEKVETWALRH (SEQ ID N°41)
 RKKRRQRRRGGATLSALLAALRRIQRA (SEQ ID N°2)
 RKKRRQRRRCGGLETRTETWMSSEGAWKQIQQVETWALRH (SEQ ID N°3)
 5 RKKRRQRRRCGGLANKKAWLDSTKATRYLVKTESWILRN (SEQ ID N° 4)

Group 2: synthetic peptides that induce cell death of adenocarcinoma cell lines: a demonstration of potency of these peptides on adenocarcinoma cells is shown in Example 6.

EHWSYWL RP GGGG RIEI WILRH (SEQ ID N°42)
 10 EHWSYWL RP GGGG RIAI WILRH (SEQ ID N°43)
 EHWSYWL RP GGGG GSRI EIWLRH (SEQ ID N°44)
 EHWSYWL RP GGGG GSRI AI WILRH (SEQ ID N°45)
 EHWSYWL RP GGGG SGAW KHAQ RIEI WILRH (SEQ ID N°46)
 EHWSYWL RP GGGG SGAW KHAQ RIAI WILRH (SEQ ID N°47)
 15 EHWSYWL RP GGGL LFIH FRIG CRH SRIG (SEQ ID N°10)
 EHWSYWL RP GGGG SLLFIH FRIG CRH SRIG (SEQ ID N°11)
 EHWSYWL RP GGGL LFIH FKIG CKH SKIG (SEQ ID N°48)
 EHWSYWL RP GGGG SLLFIH FKIG CKH SKIG (SEQ ID N°49)
 EHWSYWL RP GGGL LFIH FRIG SRH SRIG (SEQ ID N°50)
 20 EHWSYWL RP GGGG SLLFIH FRIG SRH SRIG (SEQ ID N°51)
 EHWSYWL RP GGGL LFIH FKIG SKH SKIG (SEQ ID N°52)
 EHWSYWL RP GGGG SLLFIH FKIG SKH SKIG (SEQ ID N°53)

Group 3: synthetic peptides that induce cell death of human umbilical vein embryonic cells (HUVECs). Demonstration of activity is shown in Example 7.

25 GG*CRGDMFG*CGG RIEI WILRH (SEQ ID N°54)
 GG*CRGDMFG*CGG RIAI WILRH (SEQ ID N°55)
 GG*CGRGDSPG*CGG RIEI WILRH (SEQ ID N°56)
 GG*CGRGDSPG*CGG RIAI WILRH (SEQ ID N°57)
 30 GG*CRGDMFG*CGG LLFIH FRIG SRH SRIG (SEQ ID N°5)

*C = cysteine engaged in cycling disulfide bridge

Thus, the invention provides means for having molecules acting positively or negatively on MPP. As shown by the inventors, above described peptides induce MMP: cell-permeable versions of these mitochondrio-active peptides are efficient inducers of $\Delta\psi_m$ loss and cell death by apoptosis. Therefore,

said new « mito-» fluorometric approach can be a useful and reliable tool for the identification of new mitochondrio-toxic or mitochondrio-protective drugs.

In addition, by using screened molecules and chimeric molecules composed of the mitochondrio-toxic domain sequences and a peptidic delivery system to induce cell death of tumoral cell lines (especially adenocarcinoma), or HUVECs, the inventors demonstrate the application of these sequences as potential mitochondrio-toxic molecules useful for designing new therapeutics molecules.

Molecules acting positively on MPP are useful for treating for example cancers and autoimmune diseases, and molecules negatively acting on MPP will display a cytoprotective effect (neuroprotection, cardioprotection and hepatoprotection, for example against acute or chronic ischemia). Such drugs may also be useful to treat metabolic disorders such as obesity and diabetes. More particularly, the molecules of the invention are useful for treating pathologies correlated with MPP or other dysfunctions including cell death.

Other characteristics and advantages of the invention are given in the following, with reference to figures 1 to 13.

Example 1: Mitochondrial analysis using flow cytometry

Purified mouse liver mitochondria are small organelles (1-2 μ m); Fig. 1A), which are detectable by flow cytometry using the $\Delta\psi_m$ -insensitive dye MitoTracker® Green. Indeed, correct gating on FSC/SSC parameter permits to select a population of events containing 95,5% of mitochondria (fig. 1B,C). FSC, which is highly correlated to cell size/volume (Salzman et al. 1990) permits to evaluate mitochondrial size, whereas the SSC parameter, which is related to granularity and refractive index, is a physical parameter correlated with mitochondrial internal structure. Most of these organelles are energetically competent since more than 90% have an elevated

$\Delta\psi_m$ as revealed through labeling with the $\Delta\psi_m$ -sensitive dye JC-1 (Fig. 1D).

Example 2: Mitochondrial swelling measurement: fixed time vs. real time FCM

5 Calcium (Ca^{2+}) acts on mitochondria as a PTP activator (opener) to induce a dose-dependent SSC decrease and FSC increase (Fig. 2 A, B). This effect is highly correlated with dose-dependent loss of absorbance at 545 nm (classical swelling; Fig. 2 B, C, D). Interestingly, SSC decrease
10 (internal structure modifications) appears to be the most sensitive parameters that can be measured after addition of low Ca^{2+} doses (Fig. 2B). Indeed SSC were detected at higher extent than classical swelling (Absorbance) until 7.5 μM (Fig. 2B). As expected these changes are reduced by pre-treatment of
15 mitochondria with the PTP inhibitor (closer) cyclosporin A (CsA; Fig. 2B). In addition, FSC/SSC ratio is highly correlated to spectrophotometric data (Fig. 2E). Altogether, those data indicates that flow cytometry could reliably evidence mitochondrial swelling by evaluating mitochondrial SSC
20 (internal structure) and FSC (size).

Real-time Flow cytometric recording of size and structure parameters can extend the potentialities of this approach. For this purpose, basal FSC and SSC characteristics of mitochondria are recorded for 20 minutes in the absence (Fig. 25 3; Ctr.) or presence of Ca^{2+} (Fig. 3; Ca^{2+}). No modifications are observed for the FSC, SSC and FSC/SSC ratio in control mitochondria. On the contrary, Ca^{2+} induces striking increase in FSC, FSC/SSC ratio, and a rapid decrease of mitochondrial structure (SSC) (Fig. 3; Ca^{2+}). Then, integral calculus can be
30 used to estimate the velocity of these morphological changes (Fig. 3D). Interestingly, the velocity of structure modifications (SSC) after Ca^{2+} addition is maximal within the first minute of treatment, suggesting that the SSC parameter is a very sensitive sensor of swelling-related modifications.

35 When treated with the protonophore carbonyl cyanide meta-chlorophenylhydrazone (CCCP), the mitochondrial population is

subjected to a low but significant granularity (SSC) decrease without detectable size (FSC) changes. Indeed, using real-time fluorimetric follow-up of the mean SSC signal and the mean forward scatter (FSC) signal, it appears that CCCP induces a 5 reduction of SSC (Fig. 4A) that cannot be detected by classical spectrophotometric absorbance analysis (Fig. 4B). Ca²⁺ induced PTP-opening is associated with a rapid and strong FSC/SSC ratio increase, which is the consequence of simultaneous FSC increase and SSC decrease. Moreover, CsA- 10 mediated inhibition of Ca²⁺-induced changes can be correctly followed by real-time FSC-SSC analysis (Fig. 4). When PTP-independent mitochondrial swelling is induced by the ion channel forming antibiotic peptide alamethicin, a simultaneous FSC increase and SSC decrease is also observed. Interestingly, 15 with this molecule, SSC variations were less rapid than FSC modifications. Alamethicin induced MMP is not inhibited by CsA pretreatment (not shown) and well correlated with spectrophotometric absorbance analysis (Fig. 4 A, B). In addition, real-time fluorimetric follow-up of FSC/SSC ratio 20 after alamethicin or Ca²⁺ treatment is sensitive and well correlated with classical spectrophotometric absorbance analysis (Fig. 4 C, D).

Example 3: Mitochondrial function assessed by real time FCM

25 The area of real-time fluorimetric analysis can be extended to $\Delta\Psi_m$ using the carbocyanine dye 5,5',6,6',-tetracholoro-1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). When purified mitochondria are loaded with JC-1 (0,11 nanomoles JC-1 / μ g protein), J-aggregate formation 30 (orange-red fluorescence) increases with time to reach a maximum after 15-20 minutes (Fig. 5). $\Delta\Psi_m$ suppression by CCCP addition induces a rapid decrease in JC-1 orange fluorescence and a slow decrease in monomeric JC-1 green fluorescence (Fig. 6). In contrast, PTP-dependant (induced by Ca²⁺) and PTP- 35 independent (induced by alamethicin) mitochondrial membranes permeabilization (MMP) provoke both a decrease in JC-1 orange

fluorescence and a significant increase in JC-1 green fluorescence. These phenomena most probably result from the dissociation of J-aggregates into JC-1 monomers. As expected, alamethicin and Ca^{2+} but not CCCP induce a rapid increase in 5 mitochondrial FSC/SSC ratio (Fig. 6E). Simultaneous real-time analysis of both SSC (swelling) and JC1-orange ($\Delta\Psi_m$ loss) decrease after Ca^{2+} treatment reveals a clear-cut correlation between these parameters. These analyses also indicate that at least in these experimental conditions, $\Delta\Psi_m$ loss does not 10 precede Ca^{2+} induced mitochondrial internal structure remodeling (Fig. 7). In contrast, CCCP treatment induced a rapid $\Delta\Psi_m$ loss, which precede a very slow and low SSC reduction. Thus simultaneous flow cytometric real-time recording of mitochondrial size/structure and $\Delta\Psi_m$ state may be 15 used as a tool to define the hierarchy of MMP events.

Simultaneous detection of SSC and JC1-orange is also a rapid method to screen and quantify effects of MMP-inhibitors. Accordingly, CsA the reference PTP-inhibitor completely 20 abolish Ca^{2+} induced SSC decrease and $\Delta\Psi_m$ loss (Fig. 8). Pre-treatment with bongrekic acid (BA), an ANT ligand which affect ANT conformation, strongly reduces Ca^{2+} induced $\Delta\Psi_m$ loss and partly affect SSC decrease. Another efficient 25 inhibitor of Ca^{2+} induced MMP is ruthenium red which prevent accumulation of Ca^{2+} into mitochondria through the pharmacological inhibition of the Ca^{2+} uniporter. Ruthenium red inhibits 80% of Ca^{2+} induced $\Delta\Psi_m$ loss and SSC decrease (Fig. 8). In contrast, alamethicin induced MMP is not affected by CsA, BA and ruthenium red.

Example 4: Mitochondrial alterations induced by peptides
30 The peptides corresponding to

ATLSALLAALRRIQRA	(SEQ ID N°1)
RKKRRQRRRGATLSALLAALRRIQRA	(SEQ ID N°2)
RKKRRQRRRCGGLETRTETWMSSEGAWKQIQKVETWALRH	(SEQ ID N°3)
RKKRRQRRRCGGLANKKGAWLDSTKATRYLVKTESWILRN	(SEQ ID N°4)
35 GG*CRGDMFG*CGGLLFIHFRIGSRHSRIG	(SEQ ID N°5)

RIEIWILRH	(SEQ ID N°6)
RIAIWILRH	(SEQ ID N°7)
RKKRRQRRGGRIEIWILRH	(SEQ ID N°8)
RKKRRQRRGGRIAIWILRH	(SEQ ID N°9)
5 EHWSYWLPGGGLLFIHFRIGCRHSRIG	(SEQ ID N°10)
EHWSYWLPGGGGSLLFIHFRIGCRHSRIG	(SEQ ID N°11)

Were tested in the mitochondrial real time FCM analysis and for their swelling properties, at doses ranging from 1 to 20 μ M. The results in terms of induction of mitochondrial changes 10 are summarized below in Table 1 :

peptide	Significant FSC/ SSC increase	More than 50% $\Delta\Psi_m$ loss	swelling
SEQ ID N°1	+	+	+
SEQ ID N°2	ND	ND	+
SEQ ID N°3	+	+	+
SEQ ID N°4	+	+	+
SEQ ID N°5	+	+	+
SEQ ID N°6	+	+	+
SEQ ID N°7	+	+	+
SEQ ID N°8	+	+	+
SEQ ID N°9	ND	ND	+
SEQ ID N°10	+	+	+
SEQ ID N°11	ND	ND	+

Table 1: peptide induction of MMP in isolated mitochondria.

Example 5: Induction of cellular apoptosis by MMP inducers

Characterization of apoptosis induced on cultured human 15 cells by treatment with MMP inducing peptides. The example shown relates to peptide seq. ID n°3 RKKRRQRRRCGGLERTRTETWMSSEGAWKQIQKVETWALRH. Figure 9 shows the characterization of the peptide on isolated mitochondria, demonstrating dose-dependant specific swelling (A) and release 20 of Cytochrome C (B). The peptide was then tested on cell : the HeLa cells in exponential growth phase were treated with

peptide or mock treated, then analyzed by fluorescent microscopy or FACS to ascertain apoptosis. Results shown in figure 10 demonstrate induction of apoptosis in a dose and time related effect.

5 Experiments were performed similarly for peptide SEQ.ID n°4, RKKRRQRRRCGGLANKKGAWLDSTKATRYLVKTESWILRN and are described in Figures 11 and 12. These figure demonstrate *in cellula* mitochondrial destabilization by the peptides.

10 **Example 6: Induction of cellular apoptosis by MMP inducers on adenocarcinoma cells**

Cells in exponential growth phase were cultivated in the absence (Control) or presence of peptides (peptide corresponding to SEQ ID N°11 or related control peptide (not active on mitochondria as ascertained by the real time test) 15 having HWSYWLRPG-GGGGS SEQ ID N°59. Tested human cells where non-adenocarcinoma cells Jurkat and mammary adenocarcinoma MDA-MD-231 cells. 24 hours after incubation with 5 to 20 μ M peptide, cells were subjected to FACS analysis to ascertain mitochondrial apoptosis as evidenced by induction of $\Delta\Psi_m$ loss 20 and cell membrane permeability induction revealed by FACS analysis. Table 2 show a typical result, expressed as the percent of cells demonstrating evidence of apoptosis, clearly showing induction of apoptosis especially in adenocarcinoma cells.

	Jurkat cells	Adenocarcinoma MDA-MD231 cells
Control	<5	<5
EHWSYWLRPG-GGGGS	8	7
Seq. ID n°11	18	92

25 Table 2: induction of apoptosis in cultured cells by incubation with mitochondrial permeability inducing peptide. % of apoptotic cells are indicated.

Example 7: Induction of cellular apoptosis in HUVEC by MMP inducers.

Human umbilical vein endothelial cells were treated with Seq. ID N°5 or relevant control GG*CRGDMFG*CG (not found inducing MMP ion isolated mitochondria), and apoptosis was assessed in a dose and time related manner by FACS analysis.

5 Figure 13 shows the results, clearly demonstrating and apoptosis induction in HUVEC cells treated with the peptide.

MATERIAL AND METHODS

Mitochondria isolation and purification

5 Liver mitochondria were isolated from 3-4 weeks old Balb-c mice (IFFA CREDO, Saint Germain sur l'Arbresle, France). Animals were fasted overnight before being killed by cerebral dislocation. Liver mitochondria were prepared as described (Jacotot, 2000). All steps were carried out at 4°C. Finally, 10 the organelles were resuspended in 'swelling buffer', composed by 200 mM sucrose, 5 mM succinate, 10 mM Tris-MOPS (pH 7.4), 1 mM H₃PO₄, 2 µM Rotenone, and 10 µM EGTA at pH=7.4. Spectrophotometric and flow cytometric analyses were performed in this buffer. The protein content of isolated mitochondria 15 was determined by the Bradford assay.

Reagents tested on mitochondria

Mitochondrial Membrane Permeabilization (MMP) was induced by 1-30 µM Calcium (CaCl₂, MERCK), and 5 µg/ml Alamethicin (SIGMA). Ruthenium Red (SIGMA) and the PTP inhibitor 20 Cyclosporine A (CSA, BIOMOL) were added at 10 µM 4 minutes before the addition of the MMP-inducing agents. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (mClCCP, SIGMA) was used at 20 µM to completely collapse ΔΨ_m.

25 *Detection of large amplitude swelling by spectrophotometry*

Large amplitude swelling was determined with Ultrospec 3300 pro spectrofluorometer (Amersham Pharmacia Bioech, Cambridge, England) by measuring Absorbance at 545 nm (A₅₄₅) (Bernardi et al., 1992). Mitochondria were resuspended in 1 ml 30 swelling buffer at a protein concentration of 100 µg/ml. Basal absorbance was recorded for 4 minutes, MPP-inducing reagents were added to samples and large amplitude swelling was monitored by the decrease in absorbance for the following 16 minutes. Data were recorded using the Data capture software 35 (Excel, Uppsala, Sweden). Swelling curves were drawn using the Microsoft Excel Software. The positive control of swelling was

induced by a 30 μ M CaCl₂ treatment. Percentages of specific swelling were calculated as follows: $(A_{Ca^{2+}} - A_{Reagent}) \times 100 / (A_{Ca^{2+}} - A_{init})$, were $A_{Ca^{2+}}$, $A_{Reagent}$ and A_{init} correspond to the absorbance value obtained for CaCl₂- treated, reagent - treated and pre-treated mitochondria respectively.

Flow cytometric analysis of purified mitochondria.

Fluorescence-activated flow cytometry was performed using a 3-color FACSCalibur cytometer equipped with a 15 mW air-cooled 488 nm argon laser (Becton Dickinson, San Jose, CA). Mitochondria analyses were performed after appropriate settings of the Forward angle light scatter (FSC) - Side angle light scatter (SSC) detectors (see Fig. 1). Discrimination between mitochondria and cellular debris and noise background was performed by an appropriate gating on FSC-SSC scatter plots (Fig. 1) (Radcliff G, Jaroszeski MJ 1998 Methods Mol Biol 91:1-24 Basics of flow cytometry). Systematic confirmation of the validity of this gating was obtained by a labeling of mitochondria with the $\Delta \Psi_m$ -insensitive mitochondrial dye MitoTracker® Green (MTG, Molecular Probes, Eugene, OR) (Jacotot et al., 2001). Briefly mitochondria (1.5 μ g proteins) were resuspended in 400 μ l swelling buffer containing 200 nM MTG for 15 min at room temperature. The organelles were immediately analyzed on the cytometer with the CellQuest Pro software (Becton Dickinson). MTG fluorescence was collected in the FL-1 channel (530 +/- 15 nm) and analyzed in logarithmic amplifier mode, using the PMT at a voltage adjusted to 907. Data from 20,000 gated mitochondria were recorded and analyzed.

Fixed- and real-time analysis of the mitochondrial morphological status using flow cytometry

Mitochondrial size was analyzed through the light scattered by the organelles at low angle using the Forward scatter (FSC) photodiode. Signals were collected using the E-00 setting, with a logarithmic amplification gain of 5.41. Mitochondrial structure was evaluated by the light scattered in the perpendicular direction. It was assessed by the

5 sensitive Side scatter (SSC) photomultiplier tube (PMT), using a voltage of 581, and a linear amplification gain adjusted to 4.27. The morphological changes related to swelling of purified mitochondria were monitored by the increase in mitochondrial size (FSC) and the decrease in the organelle structure (SSC) using the CellQuest Pro software. The FSC/SSC ratio was calculated by the FCS Assistant software and permitted to integrate both variations in size and structure into only one parameter.

10 FSC is mainly determined by gross mitochondrial size. According to proposed models to calculate light scatter properties (derived from the Rayleigh-Debye-Gans approximation) one can suggest that changes in the refractive index of the mitochondrial matrix also affect FSC.

15 Fixed-time analyses were performed on mitochondrial samples containing 10 µg proteins in 100 µl of swelling buffer. Samples were submitted to MPP-inducing reagents for 30 min at 25°C. Samples were immediately analyzed by flow cytometry, and data from 20.000 mitochondria were recorded.

20 The percentage of specific swelling determined on forward scatter was calculated as follows;

25
$$\frac{(\text{FSC}_{\text{Ca}^{2+}} - \text{FSC}_{\text{Reagent}}) \times 100}{(\text{FSC}_{\text{Ca}^{2+}} - \text{FSC}_{\text{Co}})}$$
, were $\text{FSC}_{\text{Ca}^{2+}}$, $\text{FSC}_{\text{Reagent}}$ and FSC_{Co} correspond to the FSC values obtained for CaCl_2 - treated, reagent - treated and untreated mitochondria respectively. Similar calculation was used to determine the percentage of specific swelling on SSC and FSC/SSC ratio values.

30 Real-time experiments were performed on mitochondrial samples containing 1.5 µg proteins in 800 µl (= 2 ng / µl) of swelling buffer. Samples were acquired on the flow cytometer for 5 minutes to record basal morphological criteria. Then reagents were added to samples, and variations in light scattering were recorded for the following 15 minutes. The sample flow rate was settled to 12 µl +/- 3 µl / min for both 35 fixed- and real-time analyses.

5 Swelling curves were obtained after plotting FSC or SSC parameters versus time, and division of the time scale into discrete periods of 1 minute. The mean FSC and SSC of mitochondria from every period were calculated by the CellQuest Pro software, and were exported into the Microsoft Excel software.

Fixed- and real-time analysis of $\Delta\Psi_m$ in isolated mitochondria using flow cytometry

10 Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by 5,5',6,6',-tetracholoro-1,1,3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Molecular Probes) incorporation. This potential-sensitive cationic dye accumulates in the mitochondrial matrix according to the Nernst equation (Reers et al., 1991). JC-1 green fluorescence, associated with JC-1 15 monomers was collected in the FL-1 channel. JC-1 orange fluorescence, associated with the formation of J-aggregates was collected in the FL-2 channel (585 +/- 21 nm) (Reers et al, 1991). Both fluorescences were recorded in logarithmic amplifier mode, using the PMT at a voltage adjusted to 907 and 20 622 respectively. Spectral overlap was avoided by adjusting compensation network as follows: FL1 - 18.5% FL2 and FL2 - 25.4% FL1. The JC-1 orange / green fluorescence ratio was calculated with the FCS Assistant software.

25 Fixed-time detection of $\Delta\Psi_m$ was performed on mitochondrial samples containing 10 μ g proteins in 100 μ l of swelling buffer. Samples were submitted to MPT-inducing drugs for 15 min at 25°C, and JC-1 was added at 800 nM for 15 min at 37°C in the dark. Samples were immediately analyzed by flow cytometry, and data from 20.000 mitochondria (gated on FSC-SSC 30 parameters) were recorded. This assay permitted to quantify the percentage of highly energized mitochondria (with both orange and green fluorescence) and depolarized mitochondria (with green fluorescence only) as depicted in part C2 of Fig.1.

Real-time monitoring of $\Delta\Psi_m$ was performed on mitochondrial samples containing 1.5 μ g proteins in 400 μ l of swelling buffer. JC-1 loading was performed at 400 nM in for 15 minutes at 37°C. Then 400 μ l of swelling buffer were added, 5 and samples were acquired on the flow cytometer for 4 minutes to register basal $\Delta\Psi_m$. Then drugs were added to the sample, and $\Delta\Psi_m$ variations were recorded for the following 16 minutes. Depolarization curves were drawn as previously indicated for swelling curves; the time scale was divided into 10 discrete periods of 1 minute and the mean of JC-1 orange and JC-1 green fluorescence of the corresponding mitochondrial subsets was calculated by the CellQuest Pro software and exported into the Microsoft Excel software.

Electron microscopy

15 Mitochondria were fixed in 3.2 % glutaraldehyde (Sigma, St Quentin Fallavier, France) in 0.1 M Soerensen buffer (0.1 M phosphate, pH= 7.2) (Prolabo, Fontenay sous Bois, France), for 16 hours at 4°C. Then, mitochondria were washed one time in 0.1 M Soerensen buffer pH= 7.2 and two times in 0.1 M 20 cacodylate buffer pH = 7.2 (Prolabo). Samples were fixed in 0.1 M cacodylate buffer containing 1 % OSO₄ acid (Ubichem) for 1 hour at room temperature. After three washings in 1 % OSO₄ acid (Ubichem) in 0.2 M cacodylate buffer, mitochondria 25 were dehydrated in 30 % methanol, stained by 2 % uranyle acetate (Merck, Fontenay sous Bois, France) in 30 % methanol, and dehydrated in a series of ethanol solutions (50-100 %). Then samples were incubated in propylene oxyde for 15 min, and embedded in epoxy (Epon 812, Merck). After sectioning by a Leica ultramicrotome, samples were stained by uranyle acetate 30 and Pb-citrate (Merck). Observations were performed using a Jeol 1200EX microscope.

Detection of Caspase-3 activation and apoptosis

Caspase-3 activation was performed using a fluorescent 35 inhibitor of caspase-3 (FLICA, FAM-DEVD-FMK) from the CaspaTag TM kit (Intergen). The loss of plasma membrane integrity occurring during apoptosis of HeLa cells was determined by 7-

Amino Actinomycin D (7-AAD) incorporation (SIGMA). 20 mg/ml 7-AAD was added to cells at 37°C for 15 min. Cells were trypsinized, resuspended in PBS and immediately acquired on the FACSCalibur. FAM-DEVD-FMK related fluorescence was 5 detected using the F1-1 channel. 7-AAD fluorescence was collected in the F1-3 channel (>650nm).

Apoptosis detection by fluorescence microscopy

HeLa cells were stained by 1 μ M and Hoechst 33-342 JC-1 for 15 minutes and observed using a DM IRB inverted 10 fluorescence microscope (Leica).

Statistical analysis

Correlations were calculated by linear regression analysis. a p value < 0.05 was considered as significant. For each analysis, R^2 is indicated.

Caption of figures

Fig. 1: Cytofluorometric identification of isolated mitochondria. A. Ultrastructural observation of purified mouse liver mitochondria. A representative electron micrograph is shown. B. Flow cytometric determination of size (Forward Scatter, FSC) and granularity (Side Scatter, SSC) of liver mitochondria. Appropriate settings of FSC (logarithmic scale) and SSC (linear scale) permit to detect events with low SSC and variable FSC (region 1), and events scattered with a variable SSC and homogenous FSC (ellipsoid gate, region 2). C. Mitochondria content of each region. Mitochondrial staining with the $\Delta\Psi_m$ -insensitive mitochondrial dye MitoTracker® Green (MTG, 200 nM, black histogram) in parallel to unstained samples (Co. grey line). The percentage of labeled mitochondria (MTG+ events) is indicated for each selected region (MTG). D. Evaluation of $\Delta\Psi_m$ status in purified mitochondria. Isolated mitochondria were submitted 15 min. to the $\Delta\Psi_m$ -sensitive fluorochrome JC-1 (800 nM) and subjected to flow cytometric determination of the fluorescence in both e FL-1 and FL-2 channels. The percentage of events is indicated in each corresponding quadrants. Events located in the region 1 (defined in B.) remained unstained by MTG and JC-1 and corresponded noise background, whereas region 2 (defined in B.) corresponded to 95.5% mitochondria with a highly energetized state (90.5% JC-1 orange mitochondria).

Figure 2: Dose-response evaluation of mitochondrial swelling by fixed-time flow cytometry.

Ca²⁺ was added to purified mitochondria at the indicated doses. A. Ca²⁺ induces dose-dependent SSC decrease and FSC increase (The mean value of FSC and SSC mean are indicated in scatter plots). Note that Ca²⁺-treated mitochondrial population harbors a characteristic 'croissant-like' shape in

the right and inferior part of FSC-SSC scatter plots. **B.** Comparative analysis of Ca^{2+} -induced mitochondrial swelling analyzed by spectrophotometry and flow cytometry (FSC and SSC parameters). Mitochondria were treated 30 min. with the indicated doses of Ca in the presence or absence of 10 μM CsA. **C.** Comparative analysis of absorbance at 545nm versus FSC/SSC ratio. **D.** Linear correlation between Δ_{FSC} and ΔA_{545} . ΔA_{545} , corresponds to the difference between the absorbance of the untreated sample and the Ca^{2+} -treated sample after 30 min of monitoring. Δ_{FSC} and Δ_{SSC} correspond to the similar calculation for FSC and SSC parameters. **E.** Linear correlation between Δ_{SSC} and ΔA_{545} . Parts C and D indicate that mitochondrial swelling evaluated by both FSC increase and SSC decrease was significantly correlated with decrease in absorbance at 545nm.

15

Figure 3: Fixed-time versus Real-time detection of Ca^{2+} -induced swelling by flow cytometry.

A, C: Fixed-time analysis of mitochondria after 20 min incubation in the absence (A) or presence (C) of 30 μM Ca^{2+} .

20 **B, D:** Representative dot-plot (SSC/FSC) analysis and electron micrographs are shown. **B, D:** Real-time monitoring of FSC (left), SSC (middle), and FSC/SSC ratio (right) absence (B) or presence (D) of 30 μM Ca^{2+} . Basal level of FSC, SSC, and FSC/SSC ratio were recorded for 5 minutes. Then, 30 μM calcium were added (vertical arrow) and morphological variations were monitored for the following 15 minutes. The white line corresponds to the mean FSC, SSC and FSC/SSC values. Under each real-time dot-plot, the velocity of mitochondrial modifications is calculated ($\Delta_{\text{FSC}}/\text{min} +/ - \text{SD}$,

25 $\Delta_{\text{SSC}}/\text{min} +/ - \text{SD}$, and $\Delta_{\text{FSC/SSC}}/\text{min} +/ - \text{SD}$; $n=7$).

Figure 4: cytofluorometric real-time detection of PTP-dependant and PTP-independent mitochondrial swelling.

(A and B) Time-course of mitochondrial swelling monitored by flow cytometry and spectrophotometry. Control mitochondria, and samples treated with 20 μ M mClCCP, 30 μ M Ca^{2+} , and 5 μ M alamethicin. The CsA inhibition experiment was performed by 5 pre-treating mitochondria by 10 μ M CsA for 5 minutes prior to Ca^{2+} addition. Arrows indicate addition of the corresponding molecule. A. Mean FSC (black curve) and SSC (grey curves) values are recorded for 20 min. B. measurement of absorbance at 545nm by spectrophotometry (classical swelling assay): C. 10 Real-time monitoring of mitochondrial swelling induced by 30 μ M Ca^{2+} , assessed by the FSC/SSC ratio (black line) versus absorbance at 545 nm (grey line). D. velocity of FSC/SSC ratio modification (Black line, grey histogram) versus absorbance at 545 nm (grey line). Means and standard deviation were obtained 15 on 7 independent experiments.

Figure 5: Real time incorporation of JC-1 in isolated mitochondria.

Basal autofluorescence of mitochondria were recorded for 20 2 min. Then, mitochondria were exposed to the $\Delta\Psi_m$ -sensitive mitochondrial dye JC-1 (400 nM) followed by cytofluorometric 25 two-color analysis for 23 minutes. A. Time-dependant incorporation of JC-1. White curves correspond to the mean of JC-1 green (upper panel; FL-1) and orange fluorescence (lower panel; FL-2). Note that JC-1 orange fluorescence (J-aggregates formation) reach a plateau after 15-20 min incubation. B. Dot-plot FL-1/FL-2 analysis of JC-1 incorporation. Recorded events 30 (A.) were re-analyzed as the sum of events during the following time-periods; 0-2min (basal auto fluorescence), 2-8 min, 8-14 min, 14-20 min and 20-25 min. An appropriate quadrant permits to quantify the percentage of unstained, single stained, and double stained mitochondria.

Figure 6: Real-time monitoring of $\Delta\Psi_m$ by flow cytometry.

Real-time co-monitoring of JC-1 orange fluorescence (A. and B.), JC-1 green fluorescence (C. and D.) and FSC/SSC ratio (E. and F.). JC-1 loaded mitochondria were exposed or not (Co.) to 20 μ M mClCCP, 30 μ M Ca^{2+} , or 5 μ M Alamethicin. Time-5 Fl-2 (A.) and Time-Fl-1 (C.) dot-plots are shown. Arrows indicate the moment of drugs addition. Differences between final and initial mean values of fluorescence are indicated (Δ). Depolarization curves (B., D., and F.) are derived from corresponding dot-plot (mean of events on a per-minute basis) 10 and expressed in linear scales.

Figure 7: Co-monitoring of SSC and $\Delta\Psi_m$ by Real-time flow cytometry in Ca^{2+} treated mitochondria.

Purified mitochondria were exposed to 30 μM Ca^{2+} (A.) or 15 20 μM mClCCP (B.) followed by SSC/FSC fixed-time dot-plot analysis (1) and real-time FCM (2, 3, 4, 5). 1. Mitochondria were analyzed before (to) and after 20 min treatment with Ca^{2+} or mClCCP. The ellipsoid region (red dots) corresponds to 20 intact mitochondria and orange dots (all events outside the region) correspond to swollen mitochondria. 2. Time-SSC dot-plot analysis. 3. Time-FL-2 (JC-1 orange fluorescence) dot-plot analysis. Note that after addition of Ca^{2+} (arrows in A.), mitochondria remained red (i.e. inside the ellipsoid region defined in 1) for a few second, and rapidly became orange 25 simultaneously to internal structure modification and a $\Delta\Psi_m$ collapse. Stabilization was achieved 7 minutes after Ca^{2+} addition. 4. Comparative mean SSC and mean $\Delta\Psi_m$ (JC-1 orange fluorescence) time-response curves ($n=7$ \pm SD). 5. Correlation between SSC and $\Delta\Psi_m$ variations.

30

Figure 8: FCM analysis of PTP-dependant and PTP-independent mitochondrial membrane permeabilization.

Mitochondria were exposed 5 minutes to 50 μM BA, 10 μM Csa, or 10 μM Ruthenium red followed by 30 min treatment with

Ca^{2+} (30 μM) or alamethicin (5 $\mu\text{g}/\text{ml}$). Then, mitochondria were subjected to FCM analysis of $\Delta\Psi_m$ (JC-1 orange fluorescence) and SSC (Mean +/-SD; n=3).

5 **Fig.9. The peptidic SEQ ID N°3 induces MMP.**

A. Activity was evaluated on isolated mitochondrial and demonstrated specific swelling, as opposed to the control peptide. B. Cytochrome C release: untreated mitochondria (1), or treated with 5 μM Alamethicin (2) or 10 μM peptide seq. ID 10 N°2 during 30 minutes were assayed for cytochrome C release by western blot on the supernatant.

Figure 10: Cellular apoptosis induced by MMP modulating peptides.

15 HeLa cells were incubated for 24 or 48h with 5 or 10 μM of peptide Seq. ID n°3 or left untreated. A. Cells were then stained with JC-1 and Hoechst dyes. Observation and counting of JC-1 low cells and nuclear condensation were done by immunofluorescence microscopy (mean of 3 experiments). B. 20 Fluorescence microscopy of the cells, after 24 hours incubation with or without 5 μM peptide and staining with JC-1 dye. C. Same conditions than B, and staining with 7AAD and detection of Caspase 3 activity using FACS analysis. D. FACS analysis of 7AAD staining.

25

Fig 11. Seq ID n°4 exhibits mitochondrio-toxic activity.
Peptidic sequence was added at 5 or 10 μM on HeLa cells and apoptosis was characterized by FAX at 24 and 48 hours.

30 **Fig 12. Compared cytotoxic effects of Seq. ID n°3 and 4.**

HeLa cells were incubated for 24 or 48h with 5 or 10 μM of peptide Seq. ID n°3 or Seq ID n°4 or left untreated. A. 35 FACS analysis of 7AAD staining. B. 24 hours incubation with or without 5 μM peptides and staining with 7AAD and detection of Caspase 3 activity using FACS analysis.

Fig 13. Cytotoxic effects of Seq. ID n°5 on Human endothelial cell (HUVEC).

HUVEC cells were incubated for 24 to 96h with 5 or 30 μ M of peptide Seq. ID n°5, control peptide, or left untreated. Apoptosis was characterized by FACS analysis after 7AAD staining. A. Dose- response versus control peptide. B. Increasing incubation times with or without 10 μ M peptides and staining with 7AAD and detection by FACS analysis.

C L A I M S

1. A method for screening modulators of mitochondrial function comprising adding a compound to be tested in a purified, isolated mitochondria preparation and simultaneously using fluorimetric analysis of mitochondrial morphology, and especially real-time fluorimetric analysis, combining analysis of morphometric parameters (SSC/FSC parameters) with analysis of membrane integrity by dye fluorescence.
- 5
2. The method of claim 1, wherein the analysis of the membrane integrity is performed using the JC-1 dye to characterize mitochondrial transmembrane potential $\Delta\psi_m$ and study any change of the mitochondrial membrane permeability.
- 10
3. The method of claim 1 or 2, wherein the mechanism of mitochondrial membrane permeabilization is further characterized using pharmacological inducers or modulators of mitochondrial permeability such as Calcium, CCCP, Alamethicin, Bongrekic acid, ruthenium red, cyclosporin A, DIDS.
- 15
4. The method of anyone of claims 1 to 3, further comprising studying endogenous or xenogenic mitochondrial regulators such as Bcl-2 family members and respiratory inhibitors on PTP-related MMP.
- 20
5. The method of anyone of claims 1 to 3, further comprising identifying compounds inducing MMP or preventing MMP induced by other agents, alone or in combination.
6. The method of anyone of claims 1 to 5, further comprising designing and defining new agents aiming at modulating MMP.
- 25
7. The method of anyone of claims 1 to 6, further comprising defining and manufacturing reagents or kits of reagent to put the method in use.
8. The method according to anyone of claims 1 to 4, further comprising characterizing mitochondrial reactivity in specific mitochondria, to be used to ascertain mitochondrial function in mitochondria from various tissues of physiological or pathological status, or pathology sources such as tumors.
- 30

9. The method according to anyone of claims 1 to 4, further comprising the diagnosis or characterization of mitochondrial function in patients with diseases, and especially genetic diseases or metabolic diseases.

5 10. Agents identified by using method according to claims 1 to 4, and shown to be able to modulate MMP on isolated mitochondria.

11. Peptides according to claim 10, having MMP modulating potency, selected in the group comprising:

10	ATLSALLAALRRIQRA	(SEQ ID N°1)
	RKKRRQRRRGATLSALLAALRRIQRA	(SEQ ID N°2)
	RKKRRQRRRCGGLERTRTETWMSSEGAWKQIQKVETWALRH	(SEQ ID N°3)
	RKKRRQRRRCGGLANKKGAWLDSKATRYLVKTESWILRN	(SEQ ID N°4)
	GG*CRGDMFG*CGGLLFIHFRIGSRHSRIG	(SEQ ID N°5)
15	RIEIWILRH	(SEQ ID N°6)
	RIAIWILRH	(SEQ ID N°7)
	RKKRRQRRGGRIEIWILRH	(SEQ ID N°8)
	RKKRRQRRGGRIAIWILRH	(SEQ ID N°9)
	EHWSYWLPGGGLLFIHFRIGCRHSRIG	(SEQ ID N°10)
20	EHWSYWLPGGGGSLLFIHFRIGCRHSRIG	(SEQ ID N°11)

and their derivatives.

Said peptides advantageously comprise in the C-terminal and N terminal position a stabilizing group such as an amide alkyl or acyl group and a marker or linking group, such as the biotinyl group. They can also include L- or D- aminoacids retro-inverso, reduced peptidic backbone, or being translated into pseudo peptides.

The invention also relates to said peptides bound to a peptidic delivery system, and optionally comprising a linker 30 between said peptides and delivery system. This peptidic delivery system could advantageously being an extra cellular or intracellular targeting sequence, an antibody or a fragment thereof (ScFv). The linker can be a sequence allowing the peptide to adopt an helical structure independently of the 35 peptidic delivery system, and can be formed by 2 to 6 aminoacids such as alanine or glycine, or a disulfide bridge,

or any such means as known by a man skilled in the art considering the state of the art knowledge. Said peptide could also be modified by insertions, deletions or mutations which conserve mitochondrial modulation potency.

5 12. Peptides obtained by analogy to peptides described in claim 11, presenting features characteristics of a MMP modulating function, defined as:

- containing at least 8 amino acids, and up to 50 amino acids
- 10 - at least a part of the peptide structure is an amphipathic alpha helix,
- 2, 3 or 4 amino acids are positively charged (lysine [K] or arginine [R]) and are located on the same side of the helix,
- 15 - when the helix is projected using helical wheel representation, the R and/or K amino acids form a cluster (see figure 15),
- when added to purified, isolated, mitochondria they induce changes (ultrastructural or membrane permeability).

Example of such peptides are selected below :

RKKRRQRRRGGGAWKHAQRRIEIWILRH	(SEQ ID N°12)
RKKRRQRRRGGGAWKHAQRRIETWILRH	(SEQ ID N°13)
RKKRRQRRRGGGAWKHAQRVESWILRN	(SEQ ID N°14)
25 RKKRRQRRRGGGAWKRACRMETWILRH	(SEQ ID N°15)
RKKRRQRRRGGGAWKQIQKVETWALRH	(SEQ ID N°16)
RKKRRQRRRGGGAWRKVEKVTWALRH	(SEQ ID N°17)
RKKRRQRRRGGGAWKHAQRRIAIWILRH	(SEQ ID N°18)
AWKHAQRRIAIWILRH	(SEQ ID N°19)
30 GG*CRGDMFG*CGGRRIEIWILRH	(SEQ ID N°20)
GG*CRGDMFG*CGGRRIAIWILRH	(SEQ ID N°21)
GG*CGRGDSPG*CGGRRIEIWILRH	(SEQ ID N°22)
GG*CGRGDSPG*CGGRRIAIWILRH	(SEQ ID N°23)
Where *C = cysteine engaged in cycling disulfide bridge	
35 EHWSYWLPGGGRIEIWILRH	(SEQ ID N°24)
EHWSYWLPGGGRIAIWILRH	(SEQ ID N°25)

EHWSYWL RP GGGGGS RIEI WILRH	(SEQ ID N°26)
EHWSYWL RP GGGGGS RIAI WILRH	(SEQ ID N°27)
EHWSYWL RP GGGGGSG AWKH AQR IEI WILRH	(SEQ ID N°28)
EHWSYWL RP GGGGGSG AWKH AQR IA I WILRH	(SEQ ID N°29)
5 EHWSYWL RP GGGGLL FIHFKIGCKHSKIG	(SEQ ID N°30)
EHWSYWL RP GGGGGSLL FIHFKIGCKHSKIG	(SEQ ID N°31)
EHWSYWL RP GGGGLL FIHFRIGSRHSRIG	(SEQ ID N°32)
EHWSYWL RP GGGGGSLL FIHFRIGSRHSRIG	(SEQ ID N°33)
EHWSYWL RP GGGGLL FIHFKIGSKHSKIG	(SEQ ID N°34)
10 EHWSYWL RP GGGGGSLL FIHFKIGSKHSKIG	(SEQ ID N°35)

in which the W6 residue can be replaced by the D-aminoacid α w.
These peptide are claimed along with their derivatives as
defined in claim 10.

Figure 1

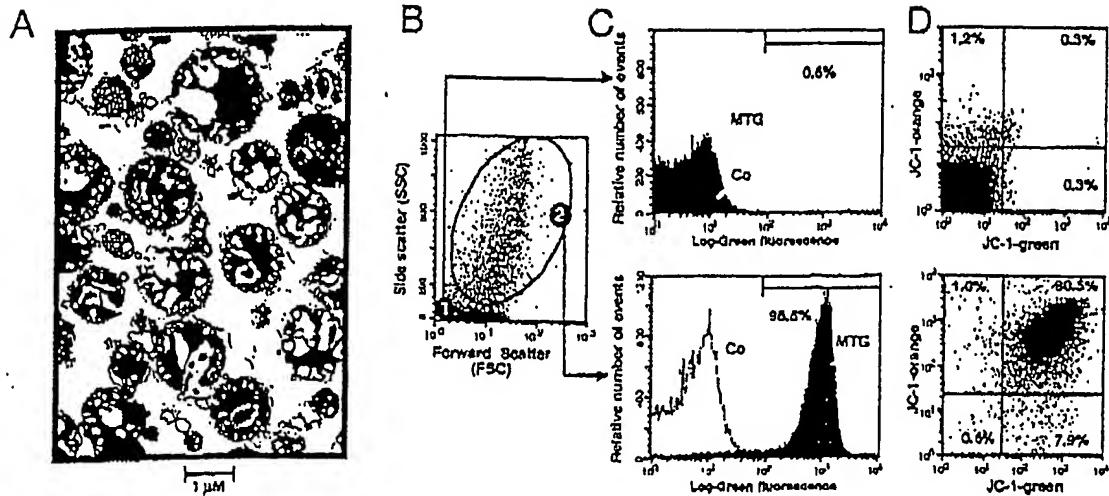
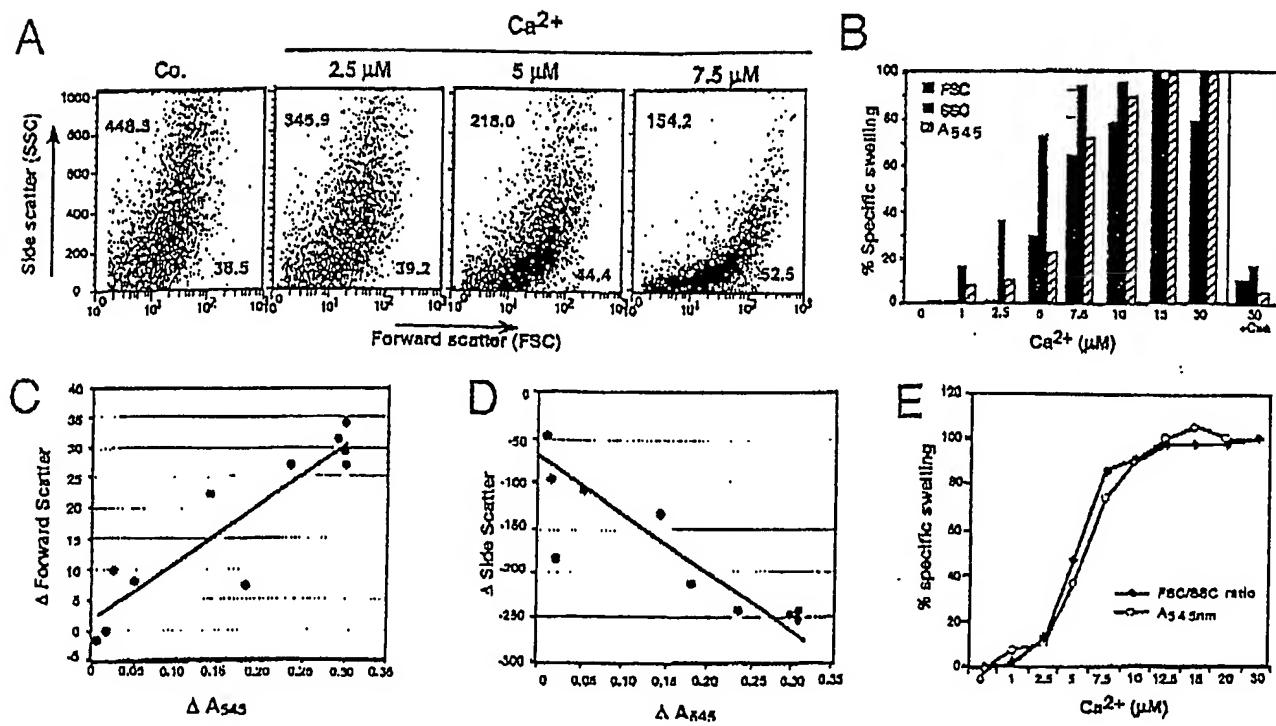


Figure 2



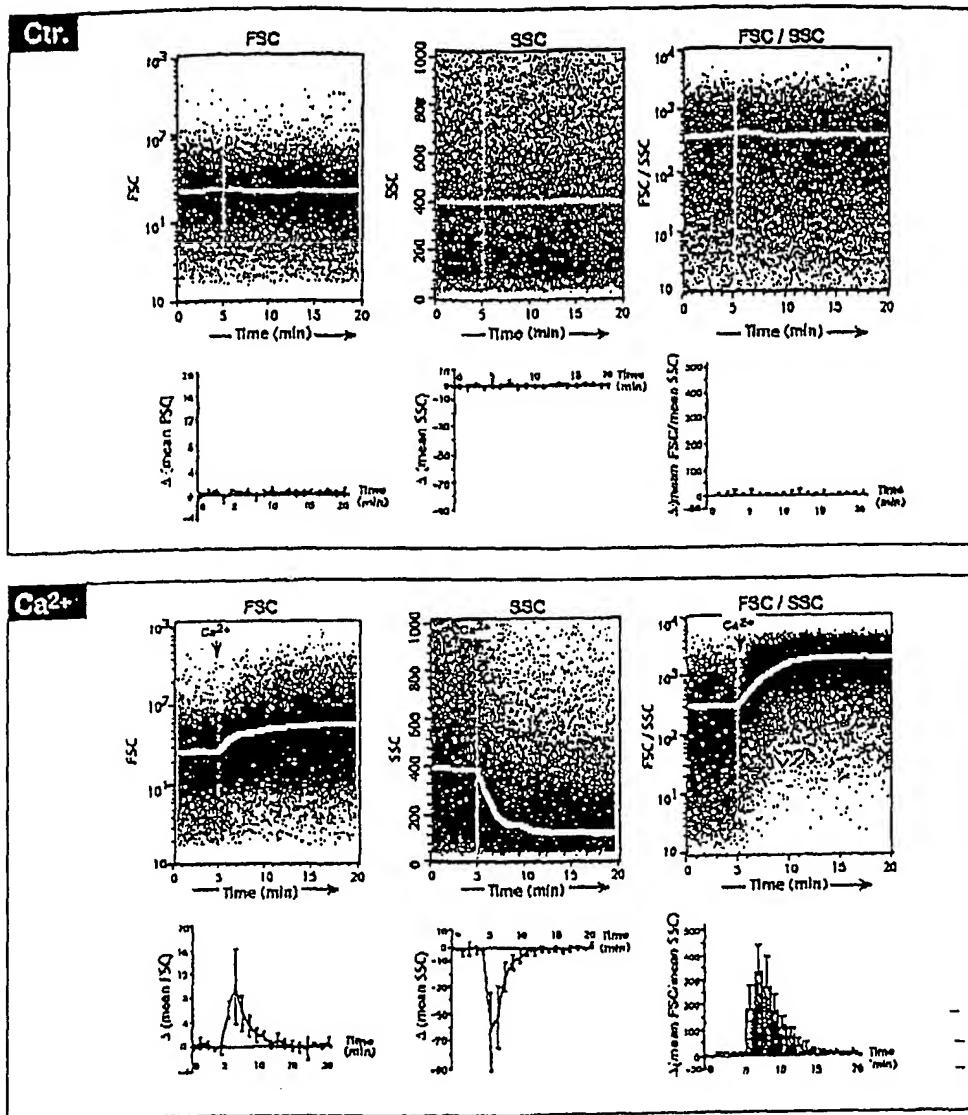


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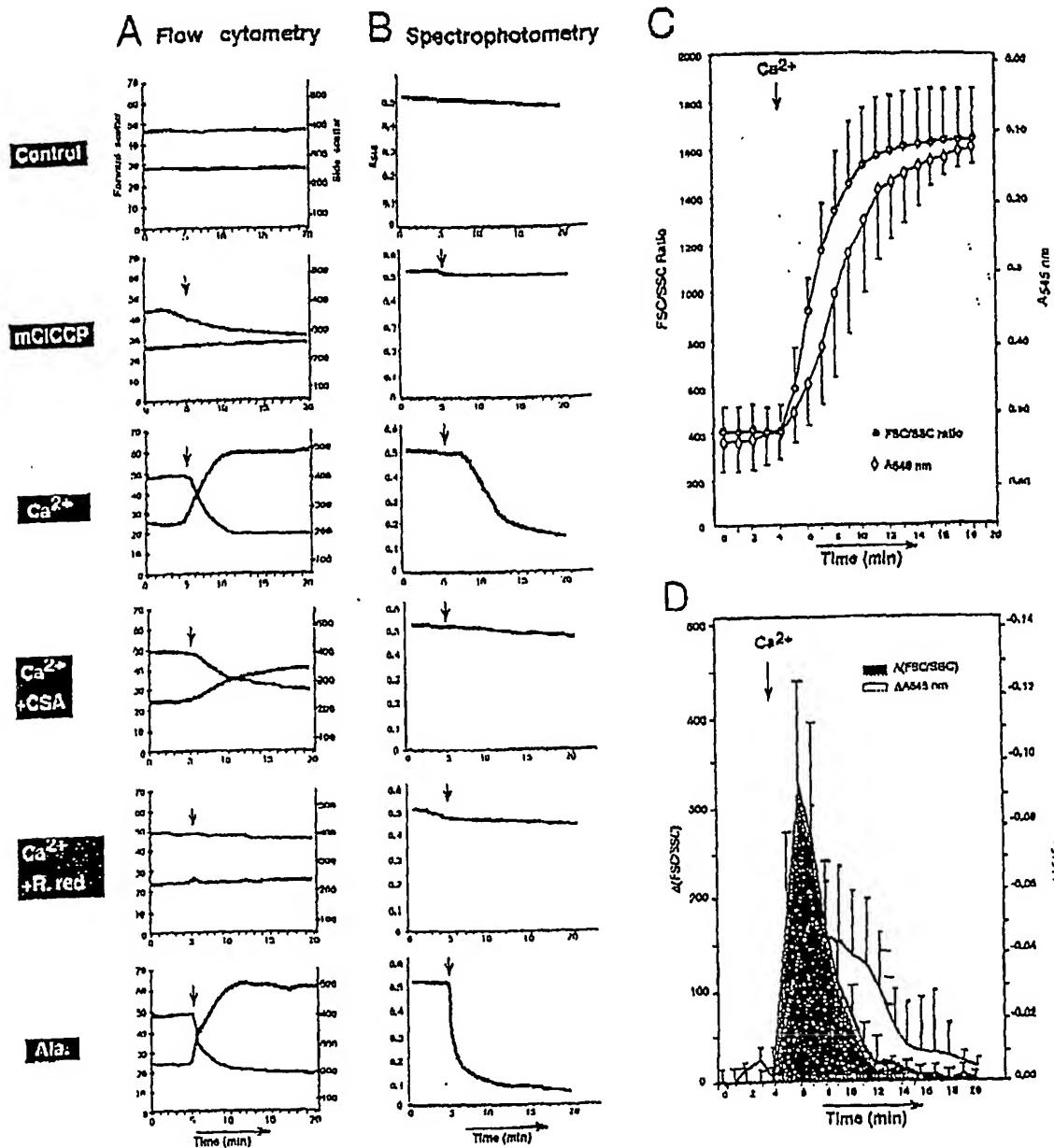
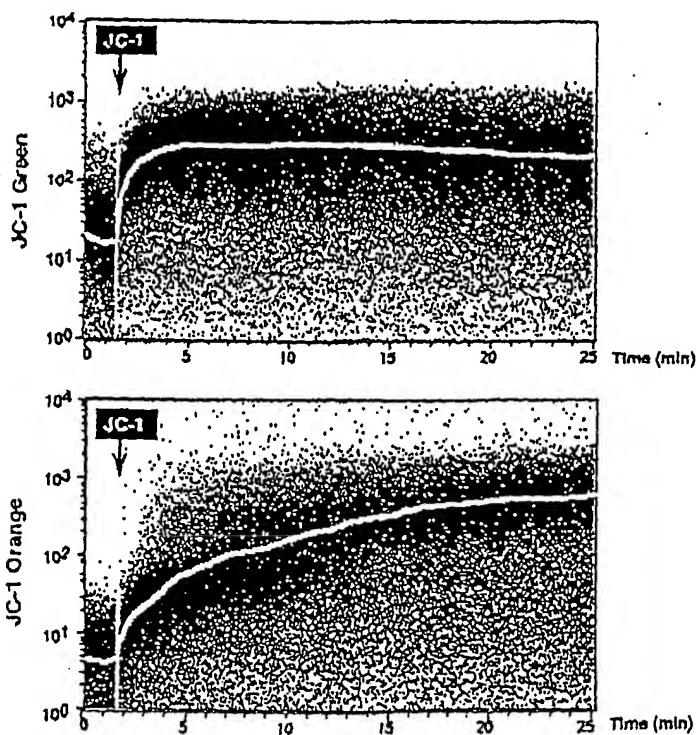


Figure 4

A



B

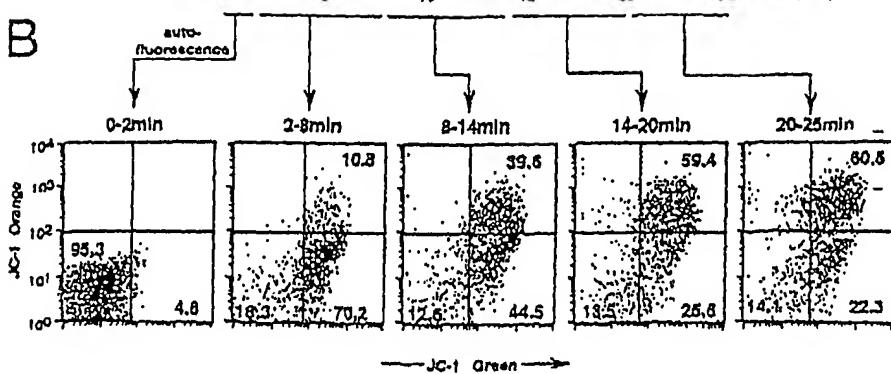


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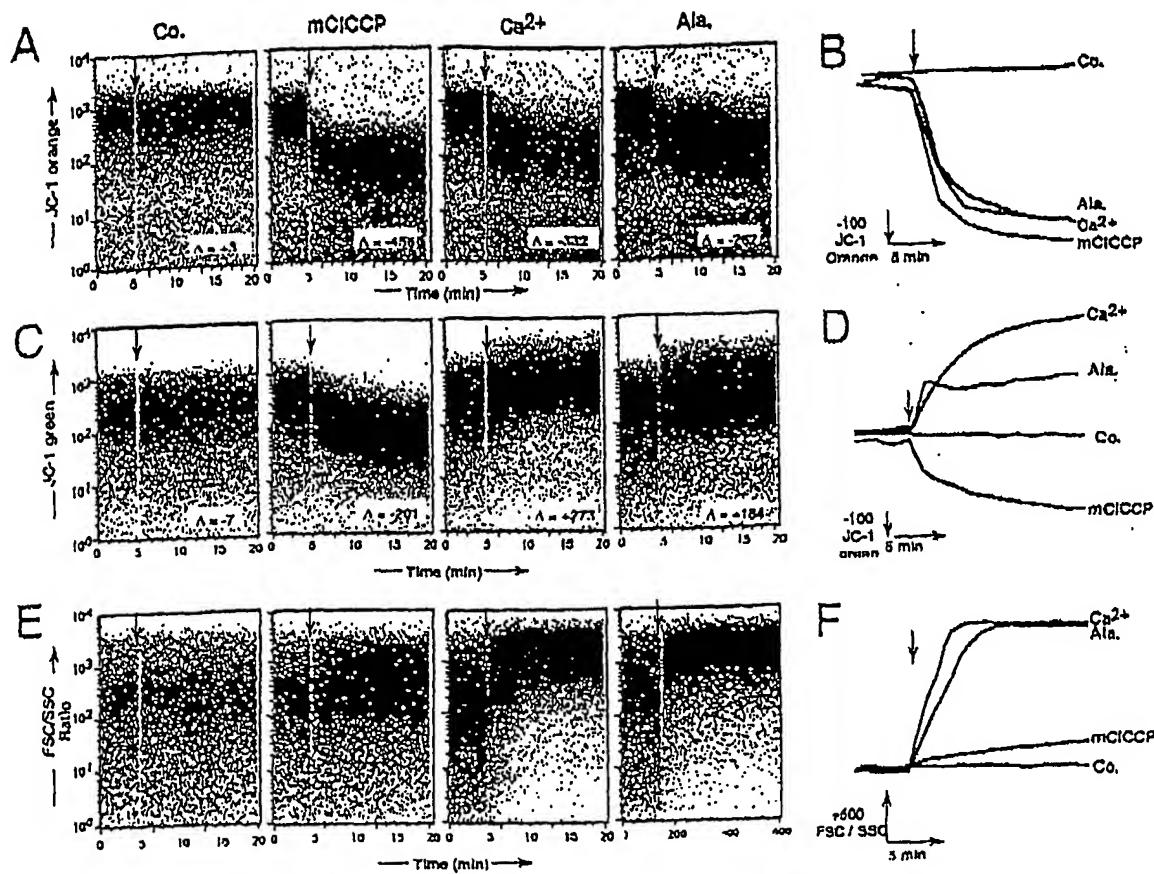


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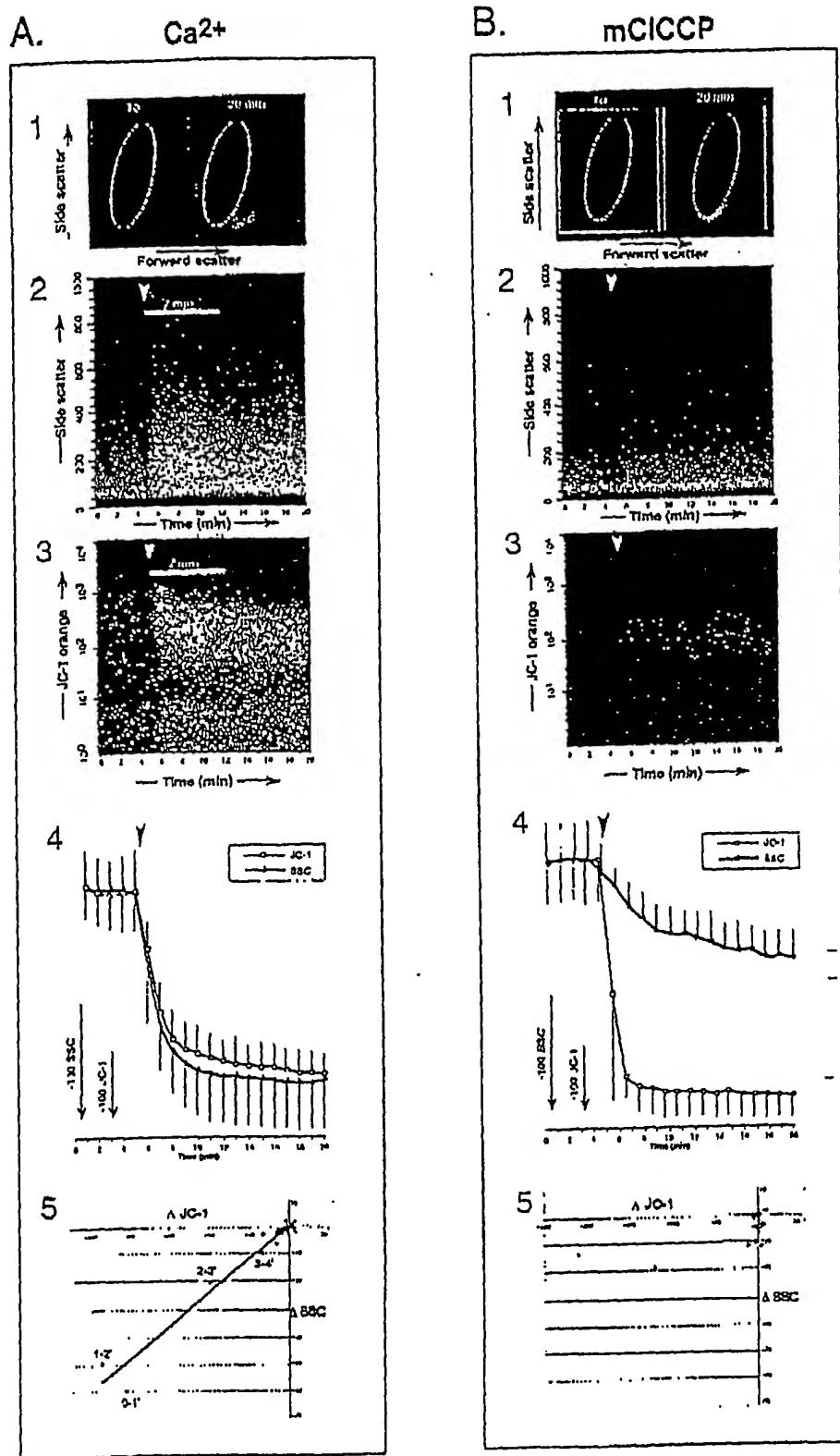
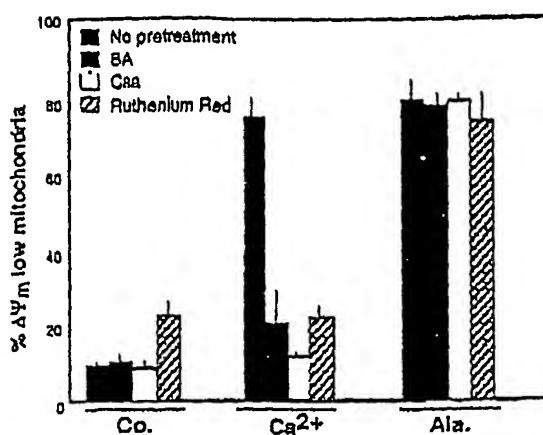


Figure 7

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A



B

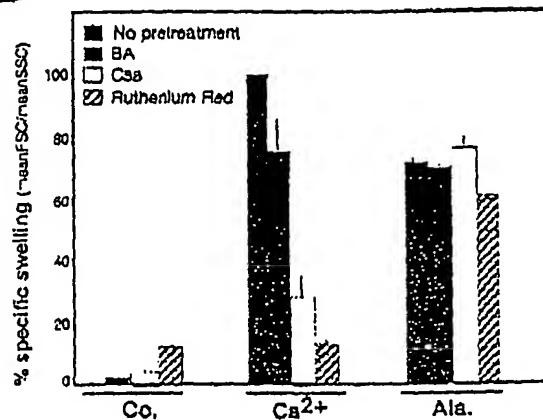


Figure 8

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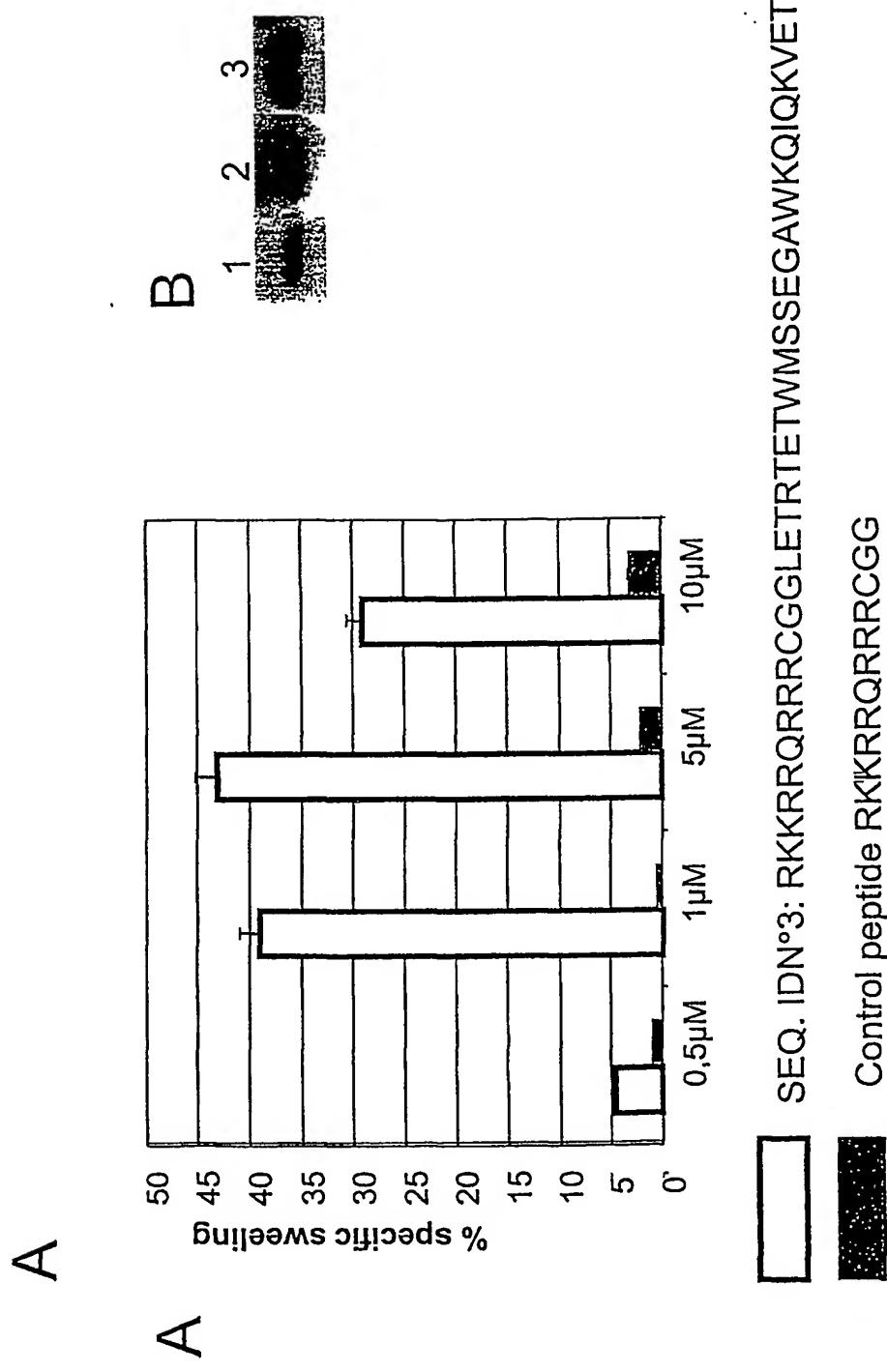
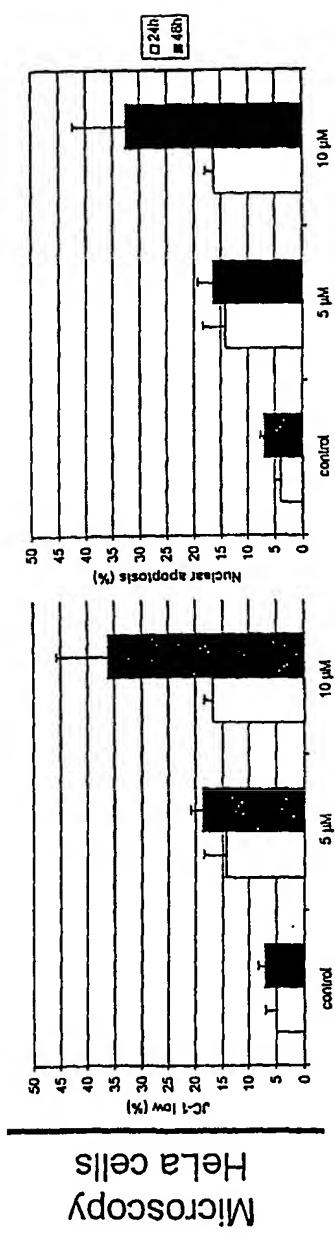
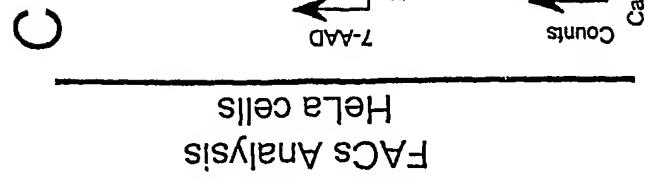


Fig. 9

B



A



D

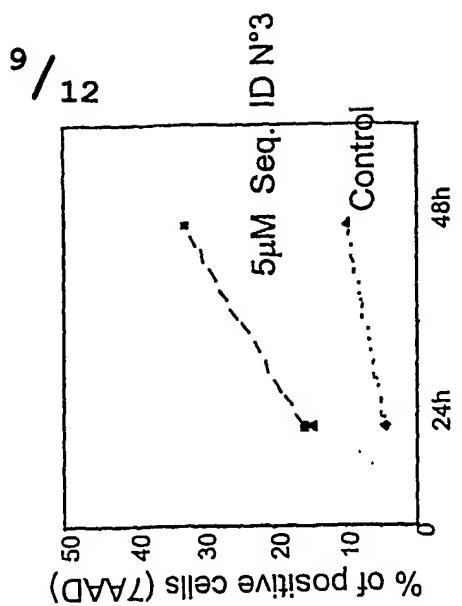


Fig. 10

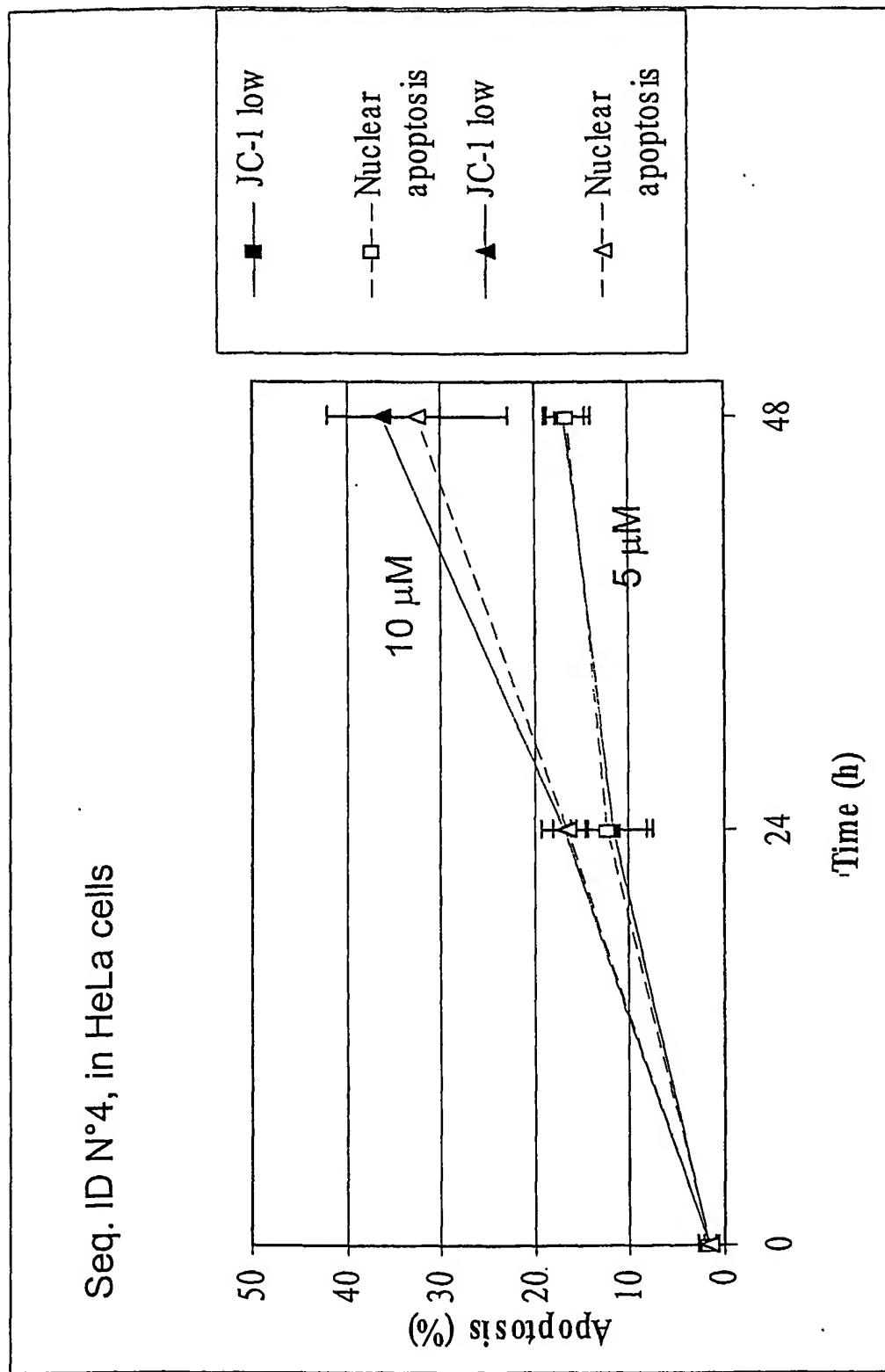
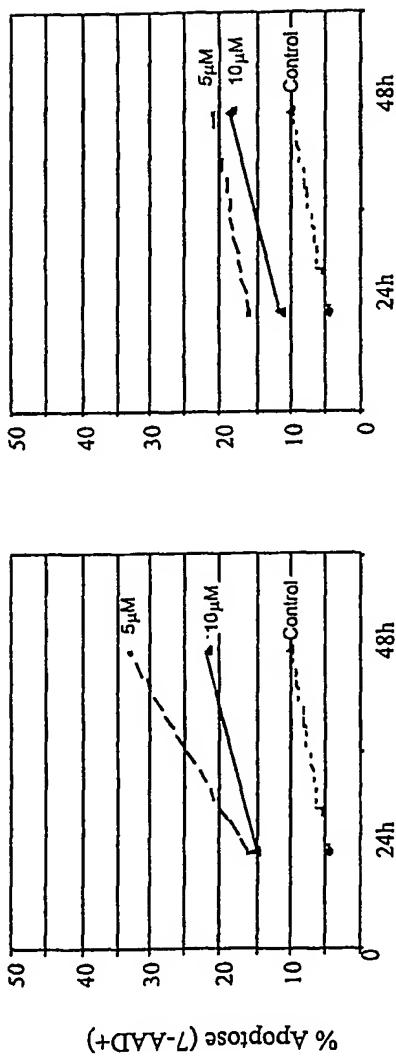
$^{10}/_{12}$ 

Fig. 11

¹¹ / ₁₂

Seq. ID N°4



Seq. ID N°3

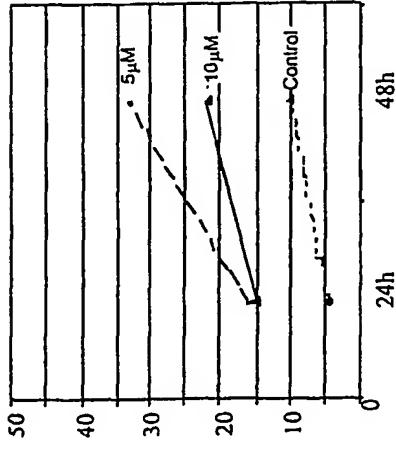
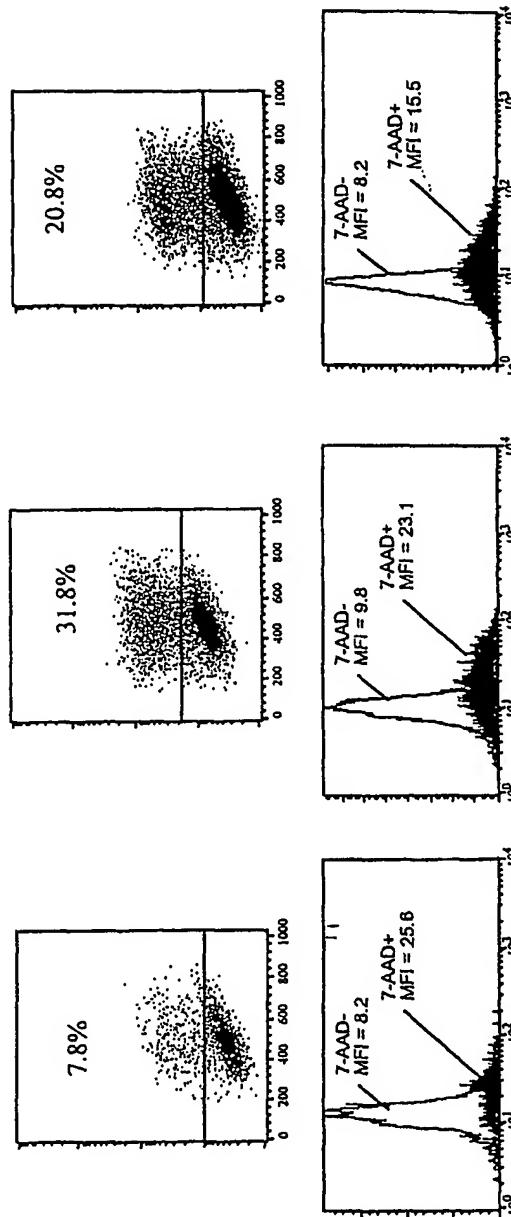


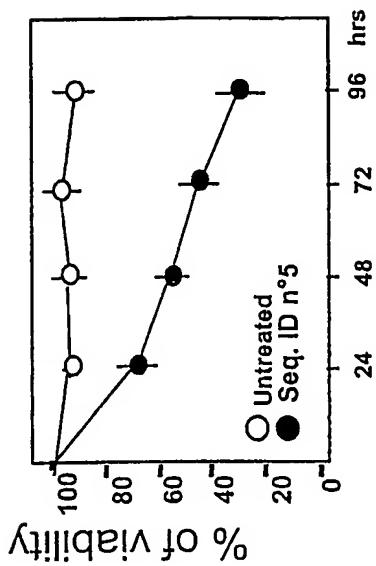
Figure 12

A**B**HeLa
5 μM Seq. ID N°4
48 h

↑ FSC
↑ SSC
↑ Counts
↑ Caspase-3

$^{12}/_{12}$

B



A

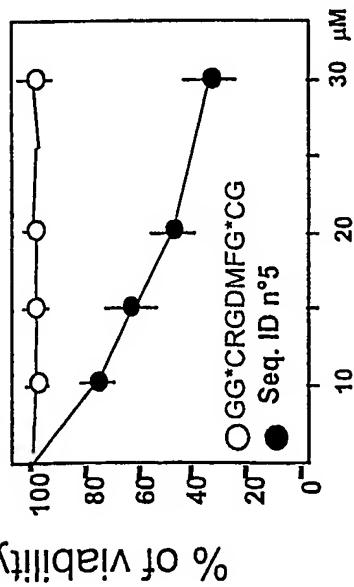


Figure 13

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<140> US 10/533 642

<141> 2005-05-03

<150> US 60/472,725

<151> 2003-05-23

<150> PCT/EP03/12056

<151> 2003-10-03

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